AD_____

Award Number: DAMD17-98-C-8003

TITLE: Preparation of a Burkholderia mallei Vaccine

PRINCIPAL INVESTIGATOR: Donald E. Woods, Ph.D.

CONTRACTING ORGANIZATION: Canadian Commercial Corporation
Ottawa, Ontario K1A 0S6 Canada

REPORT DATE: January 2002

TYPE OF REPORT: Final, Phase II

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2002	Final, Phase II (15 Dec 00 - 31 Dec 01)

4. TITLE AND SUBTITLE
Preparation of a Burkholderia mallei Vaccine

5. FUNDING NUMBERS
DAMD17-98-C-8003

6. AUTHOR(S)
Donald E. Woods, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Canadian Commercial Corporation
Ottawa, Ontario K1A 0S6 Canada

E-Mail: woods@ucalgary.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available. The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Burkholderia mallei Vaccine	70
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available.

The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms for use in humans.

Since glanders is of military significance as a biological warfare agent, the development of an effective vaccine and treatments are of particular concern. Our understanding of the disease caused by *B. mallei* is minimal, and we must move forward with these studies in order to develop new and effective vaccines and/or therapies against this organism. There is considerable dual use potential, since this disease is important in various areas of the world. Development of vaccines and treatments can, therefore, provide important items to assist the World Health Organization and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

BODY

Physical Characterization of *Burkholderia mallei* GB8 O-PS

We have been able to confirm via the use of ^{1}H and ^{13}C NMR spectroscopy that the O-PS antigen expressed by *B. mallei* GB8 is an unbranched polymer of repeating disaccharide units having the structure –3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- in which the L-6dTal*p* residues bear 2-*O*-methyl or 2-*O*-acetyl substitutions (Figure 1).

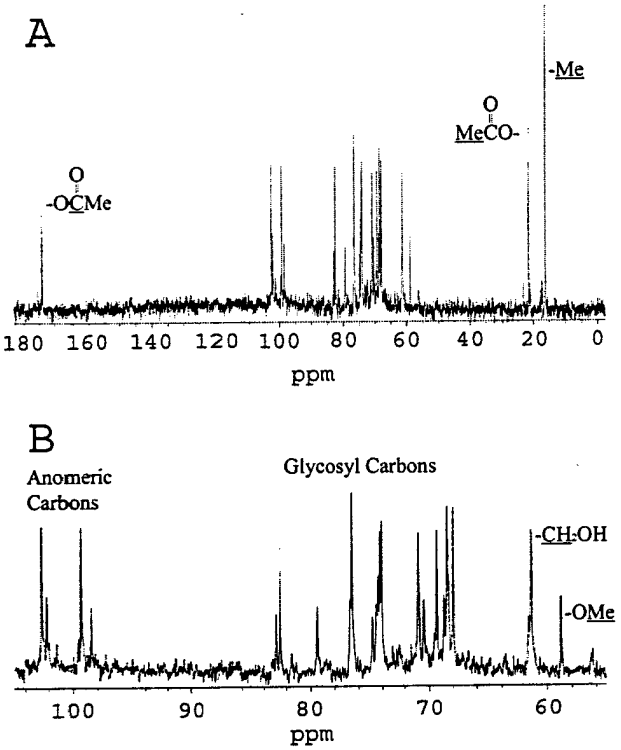


Figure 1. ^{13}C NMR spectrum of purified *B. mallei* GB8 O-PS. (A) Complete ^{13}C Spectrum of *B. mallei* GB8 O-PS. (B) Expansion of the region encompassing anomeric and glycosyl carbon signals.

Genetic Characterization of *B. mallei* GB8 O-PS

The *B. mallei* GB8 O-PS biosynthetic operon sequence has recently been annotated and submitted to GenBank (Accession Number AY028370). Comparison of the *B. mallei* GB8 operon with the previously characterized *B. pseudomallei* 1026b O-PS biosynthetic operon demonstrates a >99% sequence homology at both the nucleic and amino acid levels (Figure 2).

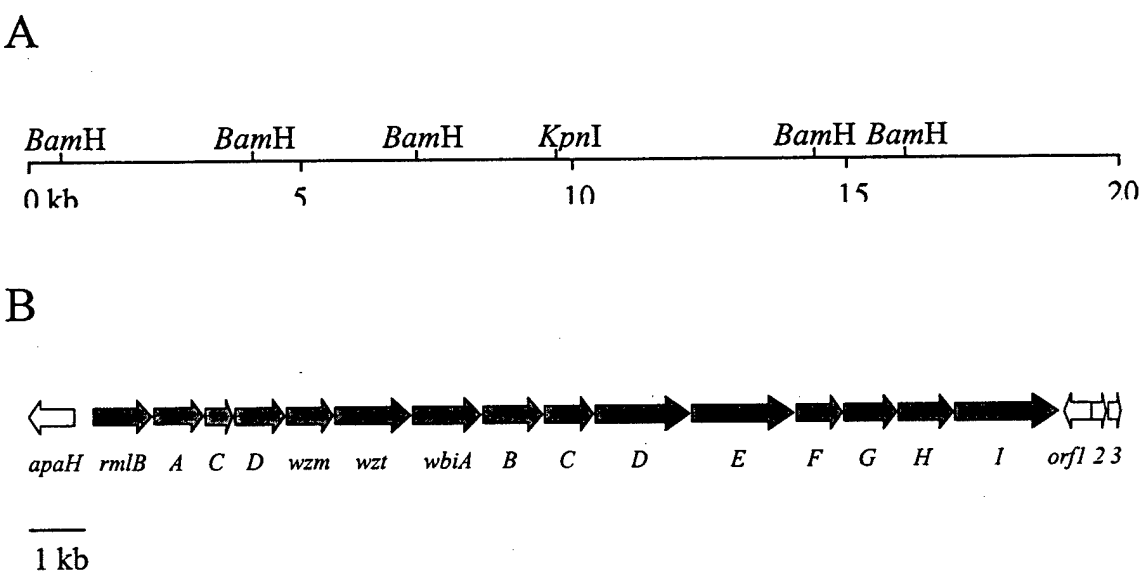


Figure 2. Physical (A) and genetic (B) map of the *B. mallei* GB8 O-PS biosynthetic operon.

Curiously, however, while the O-PS biosynthetic operons characterized in the two *Burkholderia* spp. encode for identical products, the O-PS antigens expressed by *B. pseudomallei* 1026b and *B. mallei* GB8 are similar but not identical. The difference between the two antigens is the 4-*O*-acetyl substituent present in ~33% of the L-6dTal*p* residues associated with the *B. pseudomallei* heteropolymer but not the *B. mallei* antigen. This phenomenon may be explained due to the presence of a lysogenic phage in *B. pseudomallei* strains (but not *B. mallei*) that harbors a locus with 4'-L-6dTal*p* trans-acetylase activity. Further studies will be required to confirm this hypothesis.

Immunological Characterization of *B. mallei* GB8 O-PS

Via the use of a polyclonal antiserum and a monoclonal antibody raised against *B. pseudomallei* O-PS antigens we have begun to assess the immunogenic similarities between *B. mallei* and *B. pseudomallei* O-PS molecules. These studies are important for determining whether or not a conjugate vaccine composed of only *B. pseudomallei* antigens would afford protection against *B. mallei* as well as *B. pseudomallei*. The initial results demonstrate that while the O-PS moieties expressed by the two species differ to some degree, the polyclonal antiserum is capable of reacting strongly with both (Figure 3).

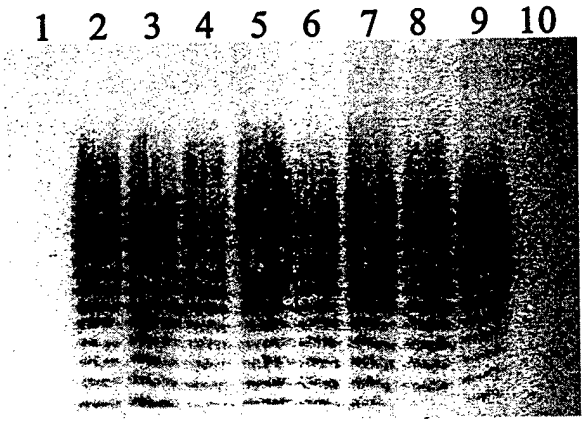


Figure 3. Western immunoblot analysis of *B. mallei* whole cell extracts reacted with rabbit polyclonal antiserum raised against *B. pseudomallei* O-PS. (1) GB3; (2) GB4; (3) GB5; (4) GB6; (5) GB7; (6) GB8; (7) GB9; (8) GB10; (9) GB11; (10) GB12.
The monoclonal antibody on the other hand does not react at all with any of *B. mallei* whole cell extracts. This phenomenon suggests that this monoclonal antibody requires the presence of 4-*O*-acetyl substituent associated with the L-6dTal*p* residues for recognition of the antigen and these are only displayed by *B. pseudomallei* strains (Figure 4).

Figure 4. Western immunoblot analysis of *B. pseudomallei* and *B. mallei* whole cell extracts reacted with a monoclonal antibody raised against *B. pseudomallei* O-PS. (1) *B. pseudomallei* 1026b; (2) *B. mallei* GB8.

B. mallei O-PS and Serum Resistance

We have recently observed that *B. mallei* GB3 and GB12 are unable to express a wild type O-PS antigen as demonstrated via Western immunoblot and SDS-PAGE analyses (Figures 3 and 5). Although the reason(s) for this phenomenon are at present unclear, it is likely that one or more of the genes in their O-PS biosynthetic operons have been

disrupted by a mobile genetic element. Sequencing and Southern blot analysis will be required to confirm this theory.

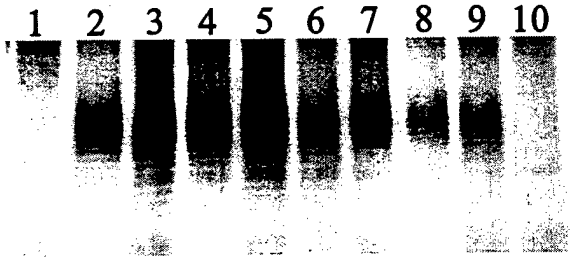


Figure 5. Silver stain analysis of *B. mallei* whole cell extracts separated by SDS-PAGE. (1) GB3; (2) GB4; (3) GB5; (4) GB6; (5) GB7; (6) GB8; (7) GB9; (8) GB10; (9) GB11; (10) GB12.

Recently, we have utilized bactericidal assays in order to determine the serum resistance phenotypes of the various *B. mallei* strains in our collection. The results of these studies demonstrated that the O-PS minus strains (GB3 and GB12) were killed in the presence of 30% normal human serum (NHS) whereas the type strain (GB8) which expresses wild type O-PS was able to survive (Figure 6). Interestingly, however, when *B. mallei* GB8 is compared to *B. pseudomallei* 1026b (positive control) a small but significant decrease in viability is observed. Further studies will be required to determine the significance of these findings.

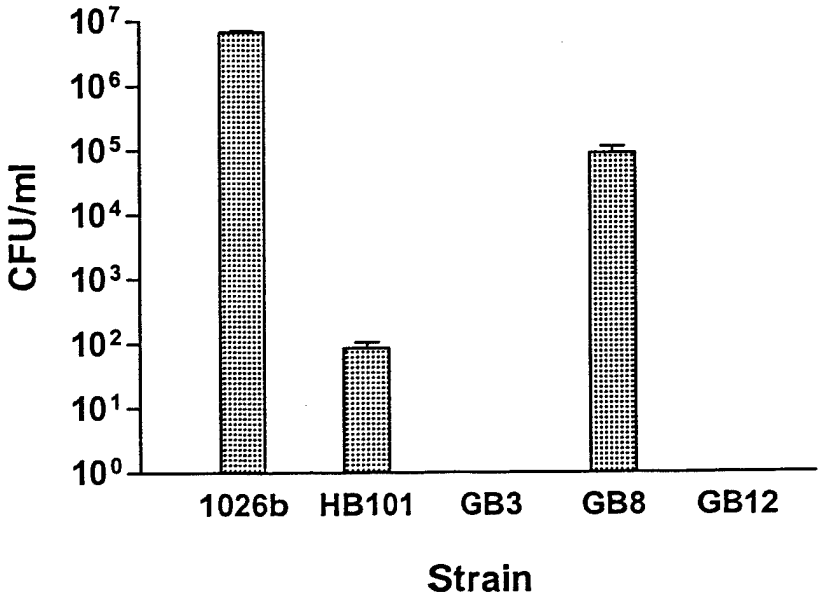


Figure 6. Survival of *B. pseudomallei* 1026b, *E. coli* HB101 and *B. mallei* GB3, GB8 and GB12 in 30% NHS. Approximately 3 x 10^6 CFU were incubated at 37°C for 2 hours in 30% NHS following which serial dilutions were plated and incubated for 48 hours. The data are the means of three separate experiments ± standard deviation.

Burlkholderia mallei LPS has been previously shown to cross react with polyclonal antibodies raised against *B. pseudomallei* LPS, however, we observed that *B. mallei* LPS cannot not bind a mAb (Pp-PS-W) specific for *B. pseudomallei* O-PS. We have identified the O-PS biosynthetic gene cluster from *B. mallei* ATCC 23344, and subsequently characterized the molecular structure the O-PS produced by this organism.

Burkholderia pseudomallei, the etiologic agent of melioidosis, is a Gram-negative bacterial pathogen responsible for disease in both humans and animals (7, 15). Previous studies have demonstrated that the O-PS II expressed by the *B. pseudomallei* is both a virulence determinant and a protective antigen (3, 6, 8, 9). Based upon these findings, O-PS II has become an important feature of the various sub-unit vaccine candidates that we are currently developing for immunization against melioidosis (1). The O-PS II moiety produced by *B. pseudomallei* is an unbranched heteropolymer consisting of disaccharide repeats having the structure –3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- in which ~33% of the L-6dTal*p* residues possess 2-*O*-methyl and 4-*O*-acetyl substitutions while the remainder of the L-6dTal*p* residues bear only 2-*O*-acetyl modifications (10, 11). Studies have also demonstrated that the non-pathogenic species *Burkholderia thailandensis* synthesizes the O-PS II antigen (2). Recently, we demonstrated that the O-antigen (O-PS) expressed by the pathogen *Burkholderia mallei* is identical to O-PS II except that it lacks acetyl modifications at the *O*-4 position of the L-6dTal*p* residues (5). Curiously, however, pair wise comparisons between the *B. mallei* and *B. pseudomallei* O-polysaccharide biosynthetic clusters failed to reveal any sequence differences that could account for the structural dissimilarities observed between O-PS and O-PS II (5, 8).

In the current studies, we used a combination of molecular and physical approaches to characterize the role of a locus, *wbiA*, thought to be involved in the acetylation O-PS II antigens (8).

Comparison of *wbiA* alleles from *B. thailandensis* and *B. pseudomallei*.

The strains, plasmids and polymerase chain reaction (PCR) primers used throughout this study are described in Table 1. Southern blot hybridizations, using a 1.37 kb *B. pseudomallei* 1026b *wbiA* PCR product, were performed in order to detect the presence of a *wbiA* homologue in *B. thailandensis* ATCC 700388. Based upon the positive results from the hybridizations (data not shown), the PCR primers wbiA-5' and wbiA-3' were used to amplify the putative *wbiA* allele from purified ATCC 700388 chromosomal DNA. The PCR product was then ligated into pCR2.1-TOPO and sequenced. A comparison between a 1239 bp open reading frame (ORF) within the cloned product and the previously characterized 1026b *wbiA* allele demonstrated sequence identities of 93.6% at the nucleotide level and 95.0% amino acids level. Further analysis of the *wbiA*

alleles demonstrated the presence of amino acid motifs that define a family of inner membrane *trans*-acylases expressed by both prokaryotic and eukaryotic species (Figure 1) (14). Based upon these results we predicted that the function of *wbiA* would be similar in both *B. pseudomallei* and *B. thailandensis*.

Phenotypic Characterization of *wbiA* null mutants.

The *wbiA* null mutants *B. pseudomallei* PB604 and *B. thailandensis* BT604 were constructed via allelic exchange using previously described methods (8). The strains were then phenotypically characterized in order to determine the effect of the *wbiA* null mutations on the synthesis of O-PS II. Silver staining of SDS-PAGE fractionated whole cell lysates demonstrated that BT604 was capable of expressing full-length lipopolysaccharide (LPS) molecules due to the presence of characteristic LPS banding patterns. The LPS was also shown to be structurally similar to that expressed by ATCC 700388 and DW503 due to its ability to react with O-PS II polyclonal antiserum (data not shown). Interestingly, however, neither the BT604 whole cell lysates or purified LPS were able to react with the O-PS II specific monoclonal antibody (mAb) Pp-PS-W suggesting that the *wbiA* locus was required for the expression of a native O-PS II antigen (Figure 2). By complementing BT604 with the broad host range vector, p31wbiA, we were able to restore the reactivity of the whole cell lysates and purified LPS with the Pp-PS-W mAb (Figure 2). Identical results were observed for the *B. pseudomallei* strains (data not shown).

Physical analysis of O-polysaccharide antigens.

The O-polysaccharides from *B. thailandensis* DW503, BT604 and BT606 and *B. pseudomallei* DD503, PB604 and PB606 were isolated and purified using previously described methods (1, 11). The purity of the carbohydrate preparations was determined to be >95% in all instances. In order to facilitate the analysis of the samples ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were recorded at 100.5 MHz and the chemical shifts were recorded in parts per million (ppm) relative to an internal acetone standard (31.07 ppm [^{13}C]; Spectral Data Services, Inc., Champaign, IL). The ^{13}C NMR spectrum of the DW503 antigen demonstrated anomeric carbon signals between 98.5 and 102.6 ppm, *O*-acetyl signals between 174.1 to 174.6 ppm ($CH_3\underline{C}O$) as well as 21.2 and 21.4 ppm ($\underline{C}H_3CO$), 6-deoxyhexose CH_3 signals at 16.0 and 16.2 ppm and an *O*-methyl signal at 58.8 ppm (Figure 3a); all of which are consistent with previously published values (11). Similar spectra were also obtained for the BT606, DD503 and PB606 samples (data not shown). In contrast, the ^{13}C NMR spectrum of the BT604 sample demonstrated anomeric carbon signals between 98.5 and 102.2 ppm, *O*-acetyl signals at 174.6 ppm ($CH_3\underline{C}O$) and 21.2 ($\underline{C}H_3CO$), 6-deoxyhexose CH_3 signals at 16.0 and 16.3 ppm and an *O*-methyl signal at 58.8 ppm (Figure 3b). Similar results were also recorded for the PB604 sample (data not shown). Based upon this data it was apparent that the O-polysaccharides expressed by BT604 and PB604 were lacking one of the two *O*-acetyl moieties associated with native O-PS II molecules.

In order to determine which of the *O*-acetyl groups was missing, a comparison of the DW503 and BT604 ^{13}C NMR spectra with the ^{13}C NMR spectrum obtained for *B. mallei* ATCC 23344 O-PS was conducted. By process of elimination we were able to conclude that BT604 and PB604 lack *O*-acetyl modifications at the *O*-2 position of the L-6dTal*p* residues because O-polysaccharides lacking *O*-acetyl substitutions only at the *O*-4 position would be expected to produce spectra consistent with that observed for the *B. mallei* O-PS antigen (Figure 4). Since *wbiA* is the only *trans*-acylase present in the O-PS II biosynthetic operon the results also suggest that a second unlinked locus is likely responsible for the *O*-acetylation of L-6dTal*p* residues at the *O*-4 position. Studies are currently underway to examine this hypothesis.

Characterization of the epitope recognized by the Pp-PS-W mAb. We have recently demonstrated that O-PS moieties expressed by *B. mallei* do not react with Pp-PS-W (5). A comparison of the O-antigens expressed by *B. pseudomallei* and *B. mallei* strains suggests that this phenomenon is due to differences in the *O*-acetylation patterns associated with the O-PS or O-PS II molecules (Figure 5). Based upon the results of the current study, it is now apparent that the mAb reacts only with –3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- polymers in which the L-6dTal*p* residues are coordinately acetylated at the *O*-2 or *O*-4 positions. Such observations are valuable because they serve to emphasize the importance of maintaining the structural integrity of O-PS II antigens during the synthesis of glycoconjugate vaccine candidates.

REFERENCES

1. Brett, P. J., and D. E. Woods. 1996. Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. *Infect Immun* 64: 2824-2828.
2. Brett, P. J., D. DeShazer, and D. E. Woods. 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *Int J Syst Bacteriol* 48: 317-320.
3. Bryan, L., S. Wong, D. Woods, D. Dance, and W. Chaoagul. 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of lipopolysaccharide from *Pseudomonas pseudomallei. Can J Infect Dis.* 5:170-178.
4. Burtnick, M., A. Bolton, P. Brett, D. Watanabe, and D. Woods. 2001. Identification of the acid phosphatase (*acpA*) gene homologues in pathogenic and non-pathogenic *Burkholderia* spp. facilitates Tn*phoA* mutagenesis. *Microbiol.* 147:111-20.
5. Burtnick, M. N., P. J. Brett, and D. E. Woods. 2002. Molecular and physical characterization of *Burkholderia mallei* O-antigens. *J Bacteriol.* 184.
6. Charuchaimontri, C., Y. Suputtamongkol, C. Nilakul, W. Chaowagul, P. Chetchotisakd, N. Lertpatanasuwun, S. Intaranongpai, P. J. Brett, and D. E. Woods. 1999. Antilipopolysaccharide II: an antibody protective against fatal melioidosis. *Clin Infect Dis* 29: 813-818.
7. Dance, D. A. B. 1996. Melioidosis, p. 925-930. *In* G.C. Cook (ed.), Manson's tropical diseases, 20th ed. W.B. Saunders Company Ltd., London, England.

8. DeShazer, D., P. J. Brett, and D. E. Woods. 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Mol Microbiol.* 30:1081-100.
9. Ho, M., T. Schollaardt, M. D. Smith, M. B. Perry, P. J. Brett, and W. Chaowagul. 1997. Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. *Infect Immun* 65: 3648-3653.
10. Knirel, Y. A., N. A. Paramonov, A. S. Shashkov, N. K. Kochetkov, R. G. Yarullin, S. M. Farber, and V. I. Efremenko. 1992. Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. *Carbohydr Res.* 233:185-93.
11. Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho. 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei. Infect Immun.* 63:3348-52.
12. Schweizer, H. P., T. Klassen, and T. Hoang. 1996. Improved methods for gene analysis and expression in *Pseudomonas* spp., p. 229-237. *In* T. Nakazawa, K. Furukawa, D. Haas and S. Silver. (ed.), Molecular biology of pseudomonads. ASM Press, Washington, D.C.
13. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology.* 1:784-791.
14. Slauch, J. M., A. A. Lee, M. J. Mahan, and J. J. Mekalanos. 1996. Molecular Characterization of the *oafA* locus responsible for acetylation of *Salmonella typhimurium* O-antigen: OafA is a member of a family of integral membrane trans-acylases. *J Bacteriol* 178: 5904-5909.
15. Woods, D. E., D. DeShazer, R. A. Moore, P. J. Brett, M. N. Burtnick, S. L. Reckseidler, and M. D. Senkiw. 1999. Current studies on the pathogenesis of melioidosis. *Microbes Infect.* 1:157-162.

Table 1. Bacterial strains, plasmids and PCR primers used in this study.

Strain, Plasmid or Primer	Relevant Characteristic(s)	Reference or Source
Strains		
E. coli		
SM10	Mobilizing strain: transfer genes of RP4 integrated into the chromosome; Km^r Sm^s	13
TOP10	High efficiency transformation	Invitrogen
B. pseudomallei		
1026b	Clinical isolate: Gm^r Km^r Sm^r Pm^r Tp^s	8
DD503	1026b derivative: Δ(*amrR-oprA*) *rpsL*; Sm^r Pm^r Gm^s Km^s Tp^s	8
PB604	DD503 derivative: *wbiA*::*dhfrIIb*-p15A oriV; Tp^r	This study
PB605	PB604 (pUCP31T); Gm^r Tp^r	This study
PB606	PB604 (p31wbiA); Gm^r Tp^r	This study
B. thailandensis		
ATCC 700388	Type strain (soil isolate): Gm^r Km^r Sm^r Pm^r Tp^s	2
DW503	ATCC 700388 derivative: Δ(*amrR-oprA*) *rpsL*; Sm^r Pm^r Gm^s Km^s Tp^s	4
BT604	DW503 derivative: *wbiA*::*dhfrIIb*-p15A oriV; Tp^r	This study
BT605	BT604 (pUCP31T); Gm^r Tp^r	This study
BT606	BT604 (p31wbiA); Gm^r Tp^r	This study
B. mallei		
ATCC 23344	Type strain (human isolate)	USAMRIID[1]
Plasmids		
pCR2.1-TOPO	TA cloning vector: ColE1 ori; Ap^r Km^r	Invitrogen
pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm^r	12
p31wbiA	1.37-kb *B. pseudomallei wbiA* PCR product cloned into the *XbaI*/*Kpn*I sites of pUCP31T; Gm^r	This study
Primers		
wbiA-5'	5'-GCTCTAGACATGAGATCGTGCTTGAGCG-3'	This study
wbiA-3'	5'-GGGGTACCGATAAAGCCAGCCCCACCGG-3'	This study

[1] United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland

FIGURE LEGENDS

Figure 1. Amino acid alignment of *B. pseudomallei* 1026b and *B. thailandensis* ATCC 700388 *wbiA* gene products. Shaded residues are highly conserved amongst the members of family of integral membrane proteins involved in the acylation of exported carbohydrates. Identity is indicated by the dots (•) while the asterisks (*) represent the sequence ends.

Figure 2. Western immunoblot analysis of purified *B. thailandensis* LPS antigens. The primary antibody used was the O-PS II specific Pp-PS-W mAb. Lane 1, DW503 LPS; lane 2, BT605 LPS; lane 3, BT606 LPS.

Figure 3. ^{13}C NMR spectra of native and mutant O-polysaccharides expressed by *B. thailandensis* strains A) DW503 and B) BT604.

Figure 4. ^{13}C NMR spectra of *B. thailandensis* and *B. malle* O-polysaccharides expanded between the region of 15-25 ppm. A) DW503, B) BT604 and C) ATCC 23344. The peaks around 16 ppm represent 6-deoxyhexose CH_3 signals while those around 21 ppm represent *O*-acetyl ($\underline{C}H_3CO$) signals.

Figure 5. Structures of A) *B. pseudomallei* O-PS II and B) *B. mallei* O-PS. In *B. pseudomallei* R' = *O*-methyl or *O*-acetyl and R''= *O*-acetyl or OH. In *B. mallei* R' = *O*-methyl or *O*-acetyl and R" = OH.

Bp MKSEIPVTVP DRFDAADVLG APQSARVADP STLANPADRL
STHDNGFGLL 50
Bt ..... E..NT..... GA.PT....R ....

Bp RLLFATMVLW DHAFPLGGFG ADPMWRLTLN QDSMGGIC[redacted]S
G[redacted]AI[redacted]F[redacted]I 100
Bt ....

Bp AKSGMRADAL Q[redacted]AWR[redacted]CV[redacted]I FPAYWAVLIV TALCVGPIIH
YVQAGTLHGY 150
Bt ....G..... .....

Bp WNAALGGPLG YITNNWRLTI GQYGINDLLR DTTPYGHSIS ESVFNGSL[redacted]T
200
Bt ....V..... ....R... .....

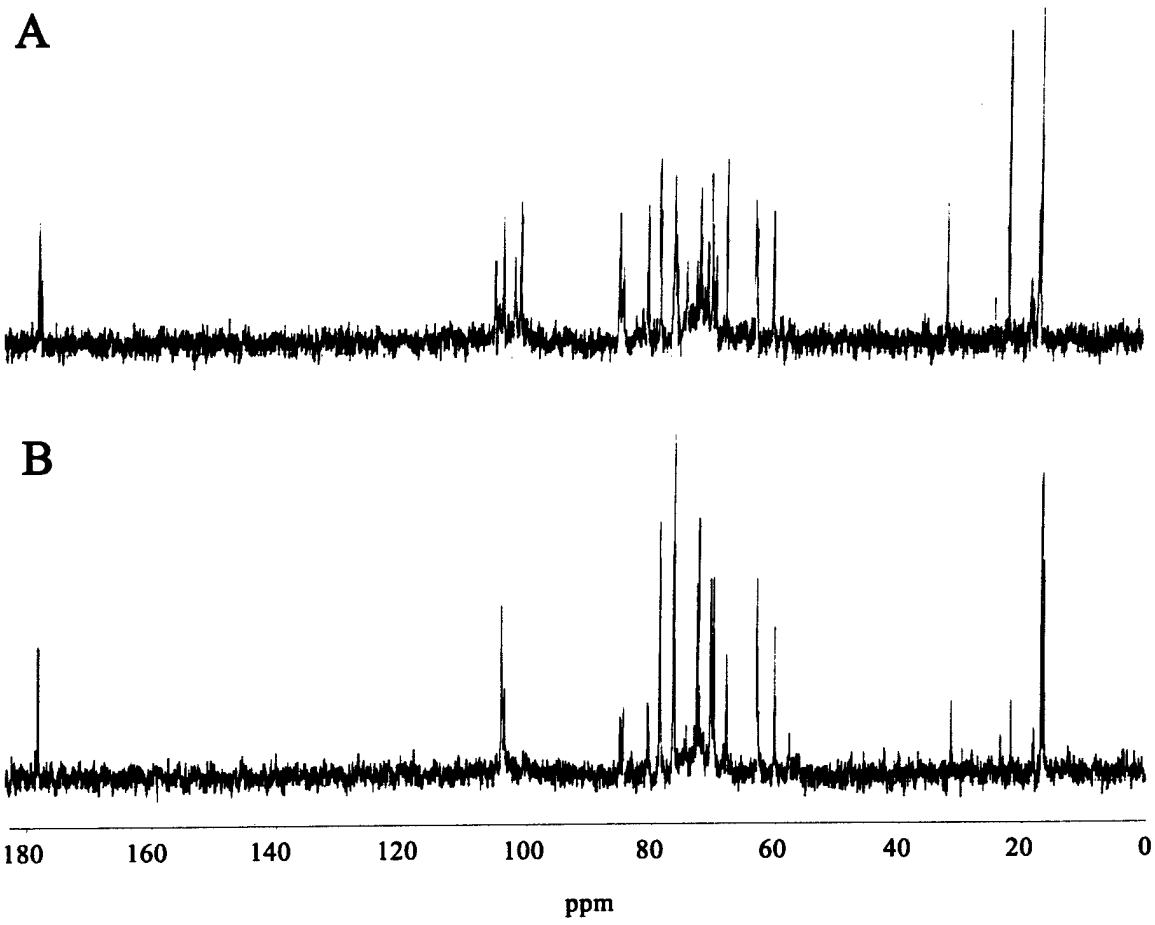
Bp [redacted]IY[redacted]AKC[redacted]VM VGLFAMFGLL TAHRRVLLAV TVVAWFVLAI
QTINPAFSAQ 250
Bt .....L .....V.... .E..P..... .G.S.L...V .....

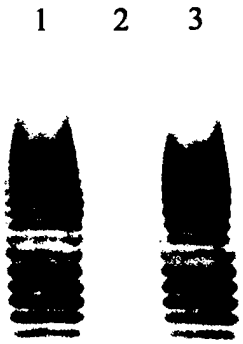
Bp LLPWAGDRHL VQYGTIFLIG SSAAAYSKSL PISDKLGAFA VVVYLISLFK
300
Bt .V..... ....I.....

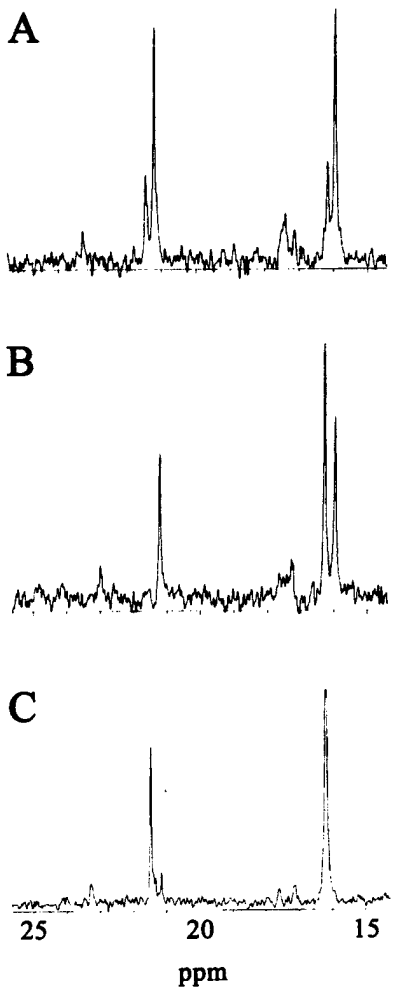
Bp GGYLLLGYPA MVYAILWLAC RLPRWARRIG SRNDYSYGIY
VFGFLVQQVL 350
Bt ....

Bp AYVGAYKYGF VFYLAASVFF TFICAWFSWH LIEKRALALK
DWGPGQGWKY 400
Bt ....

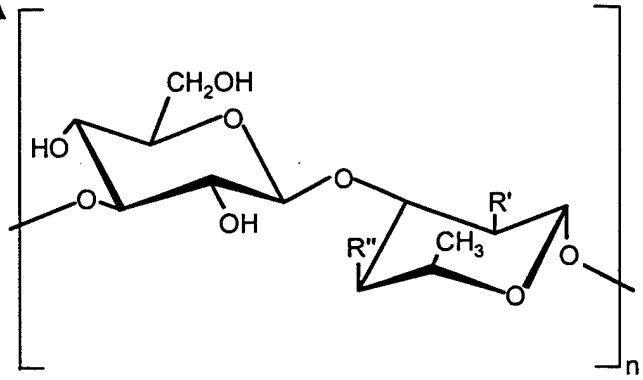
Bp CLARLTMKKE GV*
Bt ....*


A
B
180
160
140
120
100
80
60
40
20
0
ppm

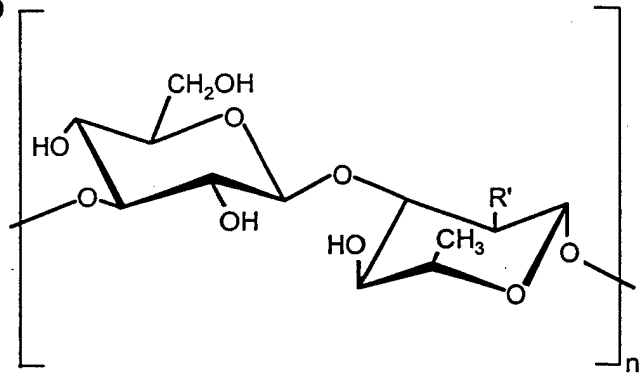

1
2
3


A
B
C
25
20
15
ppm

A



B



Studies on Capsule Production.

Animal studies have shown that capsule production by *B. pseudomallei* may contribute to persistence of the organisms in the blood. The addition 100 μg of purified capsule was shown to increase the virulence of the capsule mutant strain SR1015 in the Syrian hamster model of acute septicemic melioidosis (Table 1). As shown in Table 1, the LD_{50} value of SR1015 was similar to wild-type *B. pseudomallei* when purified capsule was added to the inoculum, and 9.0×10^2 cfu/ml of SR1015 could be isolated from the blood, nearly 1000 fold more than if the animals were infected with SR1015 only. Differences in tissue distribution between wild-type *B. pseudomallei* and the capsule mutant SR1015 in infected Syrian hamsters also suggest that the capsule contributes to persistance in the blood. As seen in Figure 2, the numbers of wild-type *B. pseudomallei* increased significantly in the blood and the spleen by 48 hours, while the numbers of SR1015 actually declined in the blood by 48 hours. The numbers of SR1015 did increase somewhat in the spleen by 48 hours, suggesting that SR1015 was readily cleared from the blood and likely sequestered in the spleen.

Capsule production by *B. pseudomallei* may be responsible for persistence in the blood through evasion of the complement cascade. The capsule mutant SR1015 has been shown to be resistant to 30% normal human serum, due to the presence of the O-PS, however, studies using purified capsule have shown that the capsule contributes to increased resistance of serum sensitive strains to the bactericidal effects of normal human serum. As shown in Figure 3, the addition of 100 μg of purified capsule increased the survival of SLR5, which lacks capsule (CPS) and O-polysaccharide (O-PS) by 100 fold. This effect was not seen with purified O-PS. Incubation of 30% serum with 100 μg of capsule for 30 minutes before the addition of the bacteria resulted in a 10,000-fold increase in survival of the serum sensitive strain SLR5 (Figure 3). The survival of SLR5 under these conditions was similar to its survival in heat-inactivated serum, which led our laboratory to conclude that the capsule was affecting the complement cascade.

Bacterial capsules have been shown to affect the bactericidal effects of the complement system through a number of mechanisms. Studies by our laboratory have shown that the *B. pseudomallei* capsule contributes to evasion of the complement system through inhibition of C3b binding to the bacterial surface. Western blot analysis of whole bacterial cells incubated in 10% normal human serum was carried out using monoclonal antibody to human C3b in order to determine if the capsule inhibited binding of C3b to *B. pseudomallei*. In the presence of capsule C3b does not appear to bind the bacterial surface effectively, but binds the bacteria readily in the absence of capsule (Figure 4). The increase in C3b deposition on the capsule mutant SR1015 may contribute to increased phagocytosis of strains lacking the capsule and as a result, increased clearance of these organisms from the blood. The increase in C3b deposition also leads to increased formation of the C3 convertase, which results in more cleavage of C3 and production of C3a, an opsonin, which attracts phagocytes. In addition, phagocytes have receptors for C3b on their surface to facilitate phagocytosis of C3b bound organisms.

Future studies on the *B. pseudomallei* capsule will be focused on further defining the role of the capsule in infection. Radiolabelled opsonophagocytosis assays are planned in order to study whether opsonization of *B. pseudomallei* strains leads to increased phagocytosis of the capsule mutant strain, thus establishing a link between C3b deposition and increased phagocytosis of capsule mutants. In addition future studies will involve studying the regulation of *B. pseudomallei* capsule using a *lux* reporter system.

B. mallei has also been shown to produce this capsule and capsule mutants of this species have been identified in this labortatory. Our laboratory plans to determine whether the role for the *B. mallei* capsule is similar to the role of the *B. pseudomallei* capsule. *B. mallei* wild type and capsule mutant strains will be tested for inhibition of C3b binding and, subsequently in the radiolabelled opsonophagocytic assays.

The combination of PCR-based subtractive hybridization, insertional inactivation, and animal virulence studies has facilitated the identification of an important virulence determinant in *B. pseudomallei*. Futher characterization of the *B. pseudomallei* capsule is essential for understanding the pathogenesis of *B. pseudomallei* infections and the development of preventative strategies for treating this disease. In addition, further characterization of the subtraction library using this methodology may lead to the identification of other potential virulence factors or protective antigens.

Figure 1. Genetic organization of the *B. pseudomallei* capsule locus.

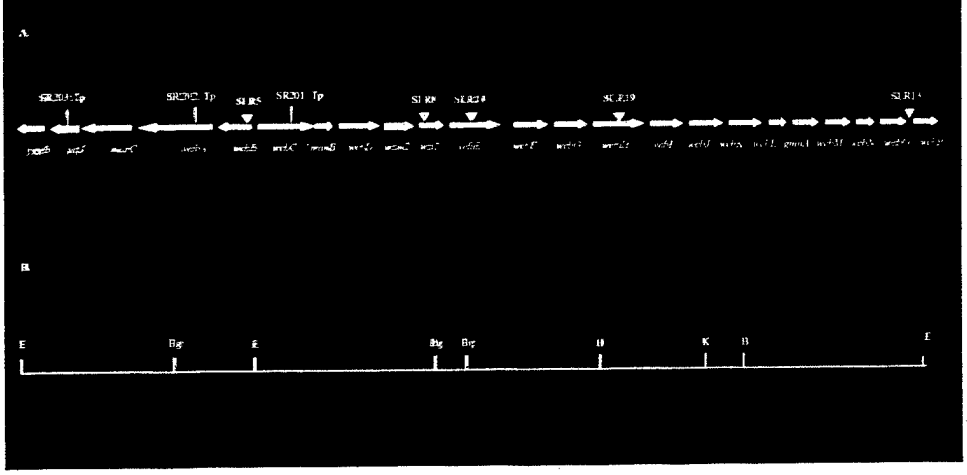


Table 1. The effect of purified capsule on the virulence of *B. pseudomallei* SR1015 in the Syrian hamster model.

Strain	**CPS added**	**LD_{50}**	**Blood bacterial count (cfu/ml)**
1026b	No	<10	5.55×10^3
SR1015	No	3.5×10^5	0
SR1015	Yes	34	9.00×10^2

Figure 2. Differences in tissue distribution between *B. pseudomallei* strains 1026b and SR1015 in the Syrian hamster model.

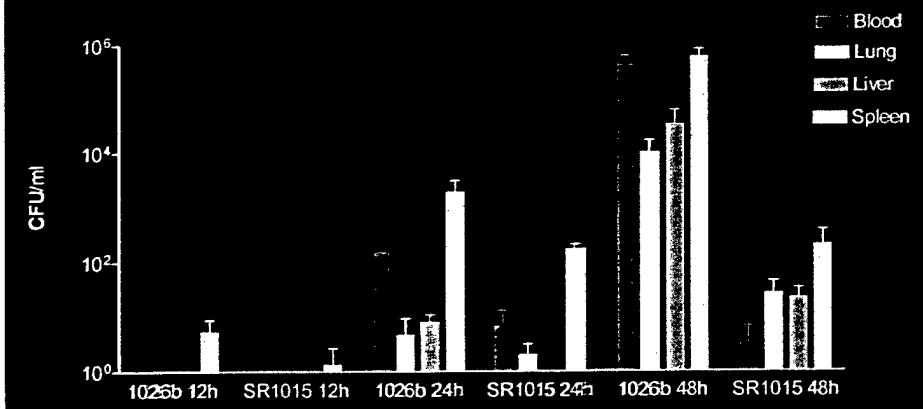


Figure 3. Purified *B. pseudomallei* capsule enhances survival of *B. pseudomallei* SLR5 in normal human serum.

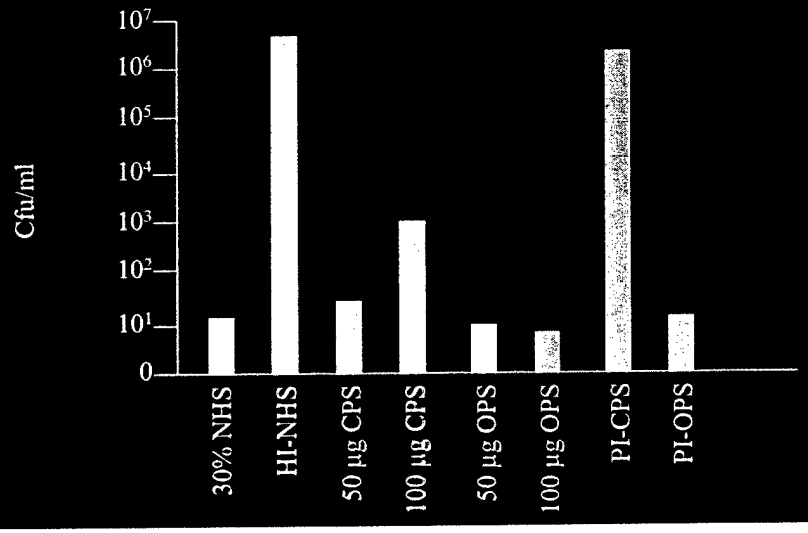
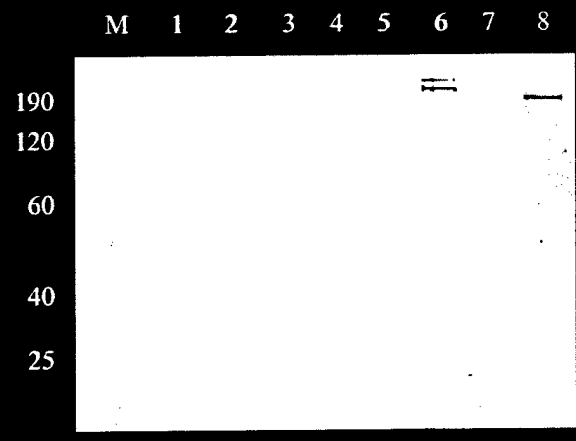


Figure 4. Western blot analysis of C3b binding in *B. pseudomallei* with monoclonal antibody to human C3bα.



Lane 1: 1026b+PBS
Lane 2: 1026b+HINHS
Lane 3: 1026b+10% NHS
Lane 4: SR1015+PBS
Lane 5: SR1015+HINHS
Lane 6: SR1015+10% NHS
Lane 7: Blank
Lane 8: 10% NHS

MICs of *Burkholderia spp.* and TOP10 carrying 3 different Beta-lactamase genes.

The sequences of B-lactamase genes from *Burkholderia mallei* and *B. pseudomallei* have been determined, and there are 3 different versions of mature B-lactamases. The proteins, PenAs, identified in *B. mallei* GB8 and *B. pseudomallei* 316a are identical, but two other mutants have been identified in clinical strains from patients who have been treated with antibiotics. The *B. pseudomallei* 316c showed selective resistance against ceftazidime and *B. pseudomallei* 392f showed decreased susceptibility to clavulanic acid inhibition. The gene sequences revealed that both mutants have single amino acid change in the active site for B-lactam antibiotics. The change at the third conserved domain, an omega loop, found in 316c may help the B-lactamase accommodate large site chain of ceftazidime, and the change from STSK of the first conserved domain to STFK found in 392f may relate to decreased susceptibility to clavulanic acid inhibition.

The MICs of 9 different antibiotics for *Burkholderia mallei* and *B. pseudomallei* and TOP10 carrying different clones were determined along with the enzyme kinetic study for these antibiotics. The results were shown in table 1 and 2, respectively

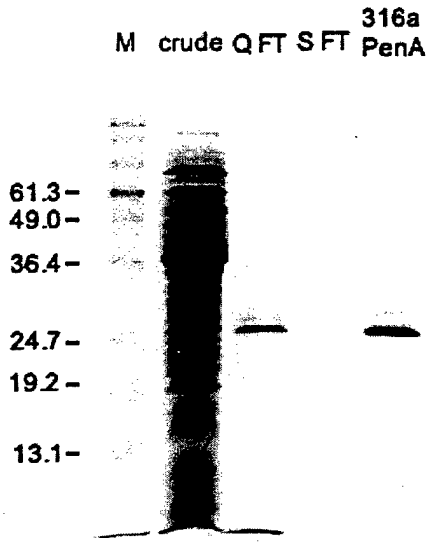


Figure 1. The 14 % SDS-PAGE shows the purified 316a PenA B-lactamase (29 kDa). M, standard protein marker in kDa; crude, crude periplasmic proteins; Q FT and S FT, Flow through from Q-sepharose and MonoS column, respectively; 316a PenA, purified 316a PenA eluted from MonoS column.

Table 1. The MICs of various B-lactams including a clavulanic acid inhibitor.

22 h 10^4CFU inocculation	Ampicillin	Amoxicillin	Amox/Clav.	Cefazolin	Ceftriaxone	Cefoxitin	Ceftazidime	Aztreonam	Imipenem

P. aeruginosa ATCC 27853	>256	>256	>128	>256	8	>128	1	4	2
S. aureus ATCC 29213	256	256	>128	>256	4	>128	8	>256	1
E. coli ATCC 25922	2	4	4	2	<1	2	0.25	<1	<0.125
TOP10	4	4	4	2	<1	4	0.25	<1	0.25
TOP10 (p316a31T)	>256	>256	128	256	4	4	2	8	0.25
TOP10 (p316c31T)	256	>256	>128	>256	4	>128	4	2	1
TOP10 (p365a31T)	>256	>256	32	256	16	8	4	8	0.25
TOP10 (p365c31T)	256	>256	32	128	16	8	4	8	0.25
TOP10 (p392a31T)	128	>256	8	128	4	8	2	8	0.25
TOP10 (p392f31T)	128	>256	16	64	4	8	1	8	0.25
GB8	64	128	8	>256	8	>128	1	32	0.25
316a	64	128	8	>256	8	>128	4	32	0.5
316c	64	128	8	>256	8	>128	64	32	0.5
365a	>256	>256	128	>256	128	>128	16	>256	2
365c	64	128	128	>256	128	>128	16	>256	0.5
392a	64	128	16	>256	4	>128	2	32	0.5
392f	64	128	32	>256	8	>128	4	64	0.5

* Amoxicillin/Clavulanic acid combination is at 8:1 ratio.

Enzyme kinetic analysis

Three different B-lactamases of 316a, 316c and 392f clones were purified using Q-sepharose fast flow in 16/20 column and MonoS HR 5/5 in FPLC (Amersham Pharmacia). The enzyme purity was assessed by 14 % SDS-PAGE (figure 1). The kinetic analysis was performed in Beckman DU640 spectrophotometer using kinetic analysis program. The enzymatic reactions were mixed to the total volume of 500 ul in 5 or 10 mm quartz cuvettes. The 316c PenA was not completely analyzed due to the difficulty in protein expression. Further optimization for 316c, PenA expression is needed to yield undetermined parameters.

Table 2. The kinetic parameters of 3 different PenAs for various B-lactams.

Substrate	PenAs	*Vmax* (nM/sec)	*Km* (M)	*kcat* (sec^{-1})	*kcat/Km* ($nM^{-1}.sec^{-1}$)
Nitrocefin	316a	159.28±7.4	10.99±2.1	4.91	446.42
	316c	144.39±3.8	12.94±1.5	ND	ND
	392f	351.61±7.2	38.06±2.1	46.22	1214.37
Ampicillin	316a	1316.66±114.2	126.5±29.5	0.18	1.45
	316c	ND	ND	ND	ND
	392f	4087.88±600.9	1051.0±236.7	37.16	35.35
Amoxicillin	316a	NH	2.22*	NH	NH
	316c	ND	ND	ND	ND
	392f	NH	74.61*	NH	NH
Cefazolin	316a	1024.68±106.0	59.45±17.8	21.35	359.08
	316c	114.14±4.0	9.80±1.63	ND	ND
	392f	2390.30±382.8	255.90±52.8	78.55	306.96
Cefoxitin	316a	NH	NR	NH	NH
	316c	ND	7.33*	ND	ND
	392f	NH	NR	NH	NH
Ceftriaxone	316a	1514.18±388.9	287.60±117.3	63.09	219.37
	316c	ND	ND	ND	ND
	392f	801.60±127.8	137.80±34.4	26.34	191.16
Ceftazidime	316a	NM	NR	NM	NM
	316c	ND	10.32*	ND	ND
	392f	NM	NR	NM	NM
Aztreonam	316a	1644.53±210.6	178.60±61.4	3.2	17.94
	316c	ND	ND	ND	ND
	392f	1938.80±144.1	335.80±51.9	17.63	52.49
Imipenem	316a	NH	NR	NH	NH
	316c	ND	NR	ND	ND
	392f	NH	NR	NH	NH
Clavulanic acid	316a	ND	0.611*	ND	ND
	316c	ND	17.66*	ND	ND
	392f	ND	3.18*	ND	ND

ND = not done, NM = not measureable, NH = not hydrolysable, NR = not recognizable by the enzyme, * = *Ki* from competition experiments reported as *Km*.

The MICs data from *E.coli* with *penA* clones showed rather good correlations to ones from *B. pseudomallei* (*B. mallei*). The selective resistance to ceftazidime of 316c was also observed in TOP10 with 316c clone, and the decreased susceptibility to clavulanic acid inhibition was also observed in TOP10 with 392f clone. The differences of MICs observed in GB8, 316a, 365a, 365c and 392f, although they have the same PenA

sequence, may involve in the level of protein expression, because there are some differences of DNA sequence outside the coding region of these strains. The same situation may occur in TOP10 clones that the DNA sequence outside the coding region were also included in the plasmid vectors. It should be noted that the cloned B-lactamase genes were not the only factor determining the MIC findings in *E. coli*. The property of the cell wall, the susceptibility of DD-transpeptidase (PBP) and the class C B-lactamase in *E. coli* can have effects on the MICs. However, it was clear that the cloned B-lactamase genes give resistant phenotypes to previously susceptible TOP10.

The kinetic parameters from this study showed that the single change in the active site of the enzyme could change the property of the enzyme readily. The *Vmax* values cannot be compared directly from one another, because the different concentrations of the enzymes were used. The *Km* may give some hints about the affinity between the enzyme and substrate, but the *kcat/Km* values can be compared very well between different versions of enzymes. There are three categories for the antibiotic substrates in this study. One is the good substrates, such as nitrocefin, cefazolin and ceftriaxone. The second group is poor substrates, such as ampicillin, amoxicillin and ceftazidime (for 316c). The last group is very poor substrates or non-substrates, such as ceftazidime, imipenem and probably cefoxitin. Although cefoxitin was not recognizable by 316a and 392f enzymes, the MICs of cefoxitin were rather high for *Burkholderia spp.*, in which the susceptibility of PBP may play some roles here. The selection of antibiotics that have proper targets is therefore important. Furthermore, the selective pressure from antibiotic use has a strong influence for the emergence of the mutant enzymes. The physicians may have to consider multiple drug regimens in the treatment of diseases caused by *Burkholderia spp*, in order to prevent the development of resistance.

Mini-O*phoA* insertions resulting in enhanced susceptibility to ß-lactam antibiotics in *Burkholderia mallei.*

Using pmini-O*phoA*, we sought to identify genes involved in ß-lactamase resistance in *B. mallei*. We identified two mutants that were hyper-susceptible to a variety of ß-lactam antibiotics. Cloning and sequencing of flanking DNA revealed that in one of the mutants the transposon had integrated in a region of the *B. mallei* chromosome which had strong homology to a gene involved in fusaric acid resistance in *B. cepacia* (*fusE*). The other transposon mutant did not have a integration in fusaric acid resistance genes but instead had an integration located in an unrelated region of the chromosome with no homology to sequences currently located in Genbank. PCR analysis of the ß-lactamase gene *penA* in *B. mallei*, showed that although this gene could be amplified from the parent strain, it could not be amplified from the two mutants nor could it be amplified from a *fusE* knockout strain. Preliminary pulse field gel analysis of these strains has demonstrated alterations in banding patterns between the parent and the mutant strains suggesting that the mutants have possibly undergone chromosomal rearrangements that have resulted in the loss of the *penA* gene. These experiments may illustrate the plasticity of the *B. mallei* chromosome and suggest that genetic manipulations using standard molecular biology techniques may result in alterations of the chromosome that result in phenotypes unrelated to the original mutations.

Regulation of antibiotic resistance genes using a lux promoter reporter system.

Use of a *lux* promoter reporter system can allow study of gene regulation without interference of regulatory pathways by mutations associated with transposon reporter systems such as *Tn*5-OT182. Promoters cloned upstream of the *lux* operon and situated in *trans* are effected by regulatory mechanisms in a manner similar to the native gene and can thereby offer a non-intrusive means of studying gene regulation.

We are currently using the *lux* promoter probe, pCS26, as a means of studying the regulation of antibiotic resistance genes in *B. pseudomallei* and *B. mallei*. By cloning the promoters of genes involved in aminoglycoside and ß-lactamase resistance in *B. pseudomallei* and *B. mallei* into pCS26, a variety of different environmental and chemical conditions can be easily examined for their ability to influence gene expression. As well, compounds that down regulate these antibiotic resistance mechanisms can be identified and may represent potential inhibitors of antibiotic resistance genes. It is hoped that this approach will further our understanding of how and when these genes are regulated during the cell cycle and will be useful in identifying compounds which may reduce the efficiency of these resistance mechanisms.

Tetracycline resistance in *B. thailandensis* and *B. pseudomallei.*

Treatment of patients with melioidosis often includes the use of the two closely related antibiotics tetracycline and doxycycline. Unfortunately, resistance to these drugs is well documented. Although tetracycline resistance mechanisms are well understood in many gram-negative bacteria, the mechanism of resistance in *B. pseudomallei* is not known. We have begun to examine tetracycline resistance in *B. pseudomallei* with the aim of understanding resistance mechanisms and identifying factors that may contribute to development of resistance during treatment. At present we have isolated strains of the non-pathogenic *B. thailandensis* that exhibit MICs to tetracycline 10-15 fold that of the parent strain. These strains will be mutagenised using *Tn*5-OT182 and mutants susceptible to tetracycline will be isolated. DNA flanking the transposon integration will be cloned and sequenced and genes identified by BLAST analysis. Genes involved in tetracycline resistance in *B. thailandensis* will be mutagenised in *B. pseudomallei* to confirm involvement in resistance in this organism.

Construction of a self-cloning *lux* transposon.

As a compliment to studying gene regulation via the *lux* promoter probe pCS26, we plan to develop a transposon that utilizes the lux operon as a reporter system. To date, much has been accomplished using *Tn*5-OT182 as a tool for identifying genes involved in the pathogenesis of *B. pseudomallei*. Although *Tn*5-OT182 mutagenesis can result in *lacZ* transcriptional fusions, following ß-galactosidase activity in these stains is disruptive and labor intensive. *Lux* fusions, on the other hand, can report gene activity throughout the growth cycle by non-invasive monitoring of light production. We will construct a self-cloning, *lux*- transposon by cloning the *lux* operon genes into the plasposons pTn*Mod*-

OGm and pTn*Mod*-OTp. Transcriptional fusions resulting from integration of a transposon containing *lux* genes will prove useful in monitoring gene expression throughout the cell growth cycle with minimal cell disruption and will compliment studies using the *lux* promoter probe pCS26 in *B. pseudomallei* and *B. mallei*.

Key Research Accomplishments

- We have been able to confirm via the use of ^{1}H and ^{13}C NMR spectroscopy that the O-PS antigen expressed by *B. mallei* GB8 is an unbranched polymer of repeating disaccharide units having the structure –3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- in which the L-6dTal*p* residues bear 2-*O*-methyl or 2-*O*-acetyl substitutions.
- Through the use of a polyclonal antiserum and a monoclonal antibody raised against *B. pseudomallei* O-PS antigens we have begun to assess the immunogenic similarities between *B. mallei* and *B. pseudomallei* O-PS molecules. These studies are important for determining whether or not a conjugate vaccine composed of only *B. pseudomallei* antigens would afford protection against *B. mallei* as well as *B. pseudomallei*. The initial results demonstrate that while the O-PS moieties expressed by the two species differ to some degree, the polyclonal antiserum is capable of reacting strongly with both.
- We have demonstrated that the presence or absence of O-acetyl groups on the O-PS moieties may have consequences when considering O-PS as a component of a vaccine that protects against both *B. mallei* and *B. pseudomallei.*
- The combination of PCR-based subtractive hybridization, insertional inactivation, and animal virulence studies has facilitated the identification of a surface capsule, an important virulence determinant in *B. pseudomalle* and *B. mallei*. Futher characterization of the capsule is essential for understanding the pathogenesis of *B. pseudomallei* and *B. mallei* infections and the development of preventative strategies for treating these diseases.
- We have developed a *phoA* mutagenesis procedure that should prove to be tremendously useful for the identification of *B. mallei* mutants deficient in the production of extracellular proteins involved in the virulence of these organisms. Now that we have developed the minitransposon-*phoA* system, these studies are proceeding rapidly.
- We have completed a number of studies on antibiotic resistance in *B. mallei*. We have demonstrated that the selection of antibiotics that have proper targets is important. Furthermore, the selective pressure from antibiotic use has a strong influence for the emergence of the mutant enzymes. The physician may have to consider multiple drug regimens in the treatment of diseases caused by *Burkholderia spp*, in order to prevent the development of resistance.

• Reportable Outcomes

- We have published a manuscript describing our studies on the identification of a pathogenicity island in *B. mallei* and *B. pseudomallei*. This manscript is attached as Appendix 1 and has been published in Infection and Immunity.
- We have collaborated with Dr. David DeShazer on a manuscript describing the details of the genes present in the pathogenicity island in *B. mallei*. This manuscript is attached as Appendix 2 and has been published in Microbial Pathogenesis.
- We have published a manuscript describing the molecular and physical characterization of Burkholderia mallei O-antigens. This manuscript is attached as Appendix 3 and will be published in the Journal of Bacteriology.
- We have submitted a manuscript to the Journal of Bacteriology describing a locus required for 2-0-acetylation of O-antigens expressed by *Burkholderia pseudomallei* and *B. thailandensis*. The manuscript is attached as Appendix 4.
- Dr. Schichao Ge is a postdoctoral fellow currently supported by this contract.
- We will apply for a renewal of a Canadian Institutes of Health Operating Grant based on work supported in part by this contract.

Conclusions

We have continued our studies on the purification and characterization of extracellular polysaccharides from *Burkholderia mallei*. In particular, we are tremendously excited about the use of extracellular polysaccharide components present on these organisms that may very well serve as ideal vaccine candidates. We have developed *TnphoA* mutagenesis utilizing pmini-*Tn5phoAMod-OGm*. Preliminary experiments indicate that this system appears more efficient than the pRT733(*TnphoA*), and this system has proven to be tremendously useful in generating mutants deficient in the production of extracellular proteins important in virulence. We have initiated studies using microarray analysis to examine the expression of virulence genes in *B. mallei*, and we have combined these studies with an examination of protein expression. The overall significance of the work resides in the realization that we are beginning to understand the virulence of *B. mallei*, and we are progressing towards the development of a vaccine that will protect against disease due to this organism.

Appendices

1. Burtnick, M.N., Bolton, A.J., Brett, P.J., Watanabe, D., and Woods, D.E. 2001. Identification of the Acid Phosphatase (*acpA*) Gene Homologues in Pathogenic and Non-pathogenic *Burkholderia spp.* Facilitates Tn*phoA* Mutagenesis. Microbiol. 147:111-120.

2. DeShazer, D., Waag, D.M., Fritz, D.L., and Woods, D.E. 2001. Identification of a *Burkholderia mallei* Polysaccharide Gene Cluster by Subtractive Hybridization and Demonstration That the Encoded Capsule is an Essential Virulence Determinant. Microb. Pathogen. 30:253-69.

3. Burtnick, M.N., Brett, P.J., and Woods, D.E. 2002. Molecular and Physical Characterization of *Burkholderia mallei* O-Antigens. Journal of Bacteriology. In press.

4. Brett, P.J., Burtnick, M.N., and Woods, D.E. 2002. The *wbiA* Locus is Required for 2-0-Acetylation of O-Antigens Expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*. Journal of Bacteriology. Submitted.

Microbiology (2001), 147, 111–120 Printed in Great Britain

Identification of the acid phosphatase (*acpA*) gene homologues in pathogenic and non-pathogenic *Burkholderia* spp. facilitates Tn*phoA* mutagenesis

Mary N. Burtnick, Alexandra J. Bolton, Paul J. Brett, David Watanabe and Donald E. Woods

Author for correspondence: Donald E. Woods. Tel: +1 403 220 2564. Fax: +1 403 283 5241. e-mail: woods@ucalgary.ca

Dept of Microbiology and Infectious Diseases, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada

***Burkholderia pseudomallei* and *Burkholderia mallei* are pathogens responsible for disease in both humans and animals. *Burkholderia thailandensis*, while phylogenetically similar, is considered avirulent in comparison. These three species exhibit phosphatase activity when grown on media containing chromogenic substrates such as 5-bromo-4-chloro-3-indolyl phosphate (XP). Tn*5*-OT182 mutagenesis has been utilized to isolate mutants of *B. pseudomallei* and *B. thailandensis* unable to hydrolyse XP. Sequence analysis of these mutants revealed an ORF of 1734 nucleotides demonstrating a high degree of homology to the *acpA* gene product of *Francisella tularensis*. PCR primers were designed based on the *B. pseudomallei acpA* gene sequence and were used to amplify an *acpA* homologue from *B. mallei*. The predicted amino acid sequence of *B. pseudomallei* AcpA differed from those of the predicted *B. thailandensis* AcpA and *B. mallei* AcpA by 15 and 3 amino acids, respectively. Allelic exchange was used to construct Δ*acpA* mutants in each of these *Burkholderia* spp. These mutants were shown to be devoid of phosphatase activity and have subsequently allowed for the implementation of *phoA* fusion transposon mutagenesis systems. Two such systems have been successfully utilized in *Burkholderia* spp. for the identification of several genes encoding exported proteins.**

Keywords: *Burkholderia* spp., acid phosphatase, Tn*phoA*, exported proteins

INTRODUCTION

Burkholderia pseudomallei, *Burkholderia thailandensis* and *Burkholderia mallei* are three closely related Gram-negative bacteria. *B. pseudomallei* is the causative agent of melioidosis, a disease endemic to South-east Asia and Northern Australia (Smith *et al.*, 1987) while *B. mallei* is the causative agent of glanders, an equine zoonosis (Arun *et al.*, 1999). *B. thailandensis* is an avirulent species that is genetically very similar to *B. pseudomallei* and *B. mallei*; however, it lacks at least one pathogenicity island present in these species (Brett *et al.*, 1998; Reckseidler *et al.*, 2001). Melioidosis and glanders have relatively high mortality rates and studies to elucidate the factors that contribute to their pathogenesis are necessary. *B. thailandensis* is a particularly useful laboratory tool for genetic manipulations under avirulent conditions which may contribute to the understanding of functions common to *B. pseudomallei* and *B. mallei*.

B. pseudomallei and *B. mallei* synthesize a variety of secreted enzymes (DeShazer *et al.*, 1999) and surface antigens; however, the roles of such factors in the pathogenesis of the diseases caused by these organisms remain poorly defined. To define the role(s) of particular exported proteins in pathogenesis, it is necessary to employ a system in which defined mutations can be made in genes encoding such products. The system we have chosen to investigate and implement in this study is the Tn*phoA* fusion vector system. The *phoA* gene fusion approach relies on the fact that the periplasmic bacterial

Abbreviations: AP, acid phosphatase; XP, 5-bromo-4-chloro-3-indolyl phosphate.

The GenBank accession numbers for the sequences reported in this paper are AF252862, AF252863 and AF276770.

alkaline phosphatase (PhoA) must be located extracytoplasmically for enzymic activity to occur (Taylor *et al.*, 1989; Manoil & Beckwith, 1985). Tn*phoA* utilizes a Tn5 transposon containing a truncated *phoA* gene which lacks a signal sequence; this transposon can generate *phoA* gene fusion randomly upon integration into the recipient bacterial chromosome (Taylor *et al.*, 1989; Manoil & Beckwith, 1985). If the targeted gene encodes an exported protein then the hybrid protein expressed will exhibit PhoA activity and the resulting colony will appear blue when grown on medium containing the chromogenic substrate 5-bromo-4-chloro-3-indoyl-phosphate (XP). Due to the fact that exported proteins are frequently involved in pathogenesis, this system provides a means by which the selection for the identification of virulence genes is enhanced. There are a number of instances in the literature in which Tn*phoA* mutagenesis has been used successfully for the identification of virulence factors. Some examples include involvement of OmpA in the virulence in *Escherichia coli* K-1 (Weiser & Gotschlich, 1991), identification of OMPs in the pathogenesis of *Salmonella abortusovis* (Rubino *et al.*, 1993), characterization of virulence genes of enteroinvasive *E. coli* (Hsia *et al.*, 1993), recognition that Tn*phoA* mutants in penicillin-binding proteins from *Erwinia amylovora* are avirulent (Milner *et al.*, 1993) and identification of antigens involved in colonization of *Vibrio cholerae* O139 (Bondre *et al.*, 1997).

B. pseudomallei exhibits phosphatase activity when grown on agar containing XP. To implement a *phoA* gene fusion system in *B. pseudomallei*, a strain that cannot hydrolyse XP must be utilized. It is known that some of this phosphatase activity is due to a surface-bound glycoprotein possessing acid phosphatase (AP) activity (Kanai & Kondo, 1994; Kondo *et al.*, 1996). However, the gene encoding an AP has remained unidentified prior to this study. In the present study we describe the sequence of the AP (*acpA*) gene homologues present in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. The AP activity associated with the *acpA* gene product was assessed. The inactivation of the *acpA* gene homologues and subsequent complementation confirms that the *acpA* gene product is responsible for the AP activity present in these species. In addition, strains harbouring disrupted *acpA* gene homologues were constructed and have allowed for mutagenesis using Tn*phoA* (Manoil & Beckwith, 1985) and mini-O*phoA* (Bolton & Woods, 2000) for the identification of genes involved in the production of exported proteins in these *Burkholderia* spp.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *B. pseudomallei* and *B. thailandensis* cultures were incubated at 37 °C on Luria–Bertani (LB) agar plates or in LB broth with agitation at 250 r.p.m. *B. mallei* cultures were grown at 37 °C on tryptic soy agar supplemented with 4% glycerol (TSG) or in TSG broth. Antibiotics were purchased from Sigma and Invitrogen. For *E. coli*, antibiotics were used at the following concentrations: 100 μg ampicillin (Ap) ml^{-1}, 25 μg kanamycin (Km) ml^{-1}, 25 μg chloramphenicol (Cm) ml^{-1}, 100 μg streptomycin (Sm) ml^{-1}, 15 μg gentamicin (Gm) ml^{-1}, 15 μg tetracycline (Tc) ml^{-1}, 50 μg polymyxin B (Pm) ml^{-1}, 1·5 mg trimethoprim (Tp) ml^{-1} and 25 μg zeocin (Ze) ml^{-1}. For *B. pseudomallei* and *B. thailandensis* the antibiotic concentrations used were 50 μg Km ml^{-1}, 50 μg Tc ml^{-1}, 100 μg Tp ml^{-1} and 100 μg Ze ml^{-1} unless otherwise stated. For *B. mallei* antibiotic concentrations used were 75 μg naladixic acid (Nx) ml^{-1}, 5 μg Km ml^{-1}, 5 μg Ze ml^{-1}, 15 μg Pm ml^{-1} and 5 μg Gm ml^{-1}.

Plasmids were purified using the Concert rapid plasmid miniprep system (GibcoBRL), QIAprep spin plasmid miniprep kit (Qiagen) or QIAprep midipreps for plasmid DNA (Qiagen).

Tn5-OT182 mutagenesis and screening. *B. pseudomallei* 1026b and *B. thailandensis* E264 were mutagenized with Tn5-OT182 as previously described (DeShazer *et al.*, 1997). *B. pseudomallei* conjugations were incubated at 37 °C for 8 h while those of *B. thailandensis* were incubated at 37 °C for 2 h. Transconjugants were selected for on LB agar plates containing 100 μg Sm ml^{-1} and 50 μg Tc ml^{-1} with 40 μg XP ml^{-1}. White colonies were retained for further analyses.

DNA manipulation and transformations. Restriction endonucleases and T4 DNA ligase were purchased from GibcoBRL and New England Biolabs, respectively, and were used according to the manufacturers' instructions. DNA fragments excised from agarose gels and used in cloning procedures were purified using a QIAquick gel extraction kit (Qiagen). A Wizard genomic DNA purification kit (Promega) was used for isolation of genomic DNA from bacterial strains. The DNA immediately flanking Tn5-OT182 integrations was self-cloned as previously described (DeShazer *et al.*, 1997). In brief, approximately 5 μg chromosomal DNA from Tn5-OT182 mutants was digested with restriction enzyme, boiled for 5 min and precipitated with 0·1 vol. 3 M sodium acetate and 2 vols 100% ethanol. This mixture was placed at −70 °C for at least 30 min, centrifuged and washed with 70% ethanol. The resulting DNA was air-dried, resuspended in distilled water and ligation reactions were prepared. Transformations were performed with 2–10 μl ligation mixture using chemically competent *E. coli* cells.

Phosphatase activity assays. AP activity assays were performed in triplicate using a previously described method (Kondo *et al.*, 1991b, 1996). Supernatants, periplasmic proteins and whole cells were prepared from 1 ml of overnight cultures grown at 37 °C. Supernatants were harvested and filter-sterilized through a 0·22 μm filter (Millipore) for use in supernatant assays. Whole cells were pelleted, resuspended in 1 ml 0·01 M Tris/HCl pH 8·0 and used in whole-cell assays. Periplasmic proteins were extracted using a previously described chloroform extraction method (Ames *et al.*, 1984). In a typical assay, 20 μl of the test sample, 20 μl *p*-nitrophenyl phosphate (0·2%, w/v, solution) and 160 μl 0·1 M sodium acetate buffer pH 5·5 were mixed and incubated at 37 °C for 30 min in microtitre wells. Then 100 μl 0·5 M NaOH was added and the colour was allowed to develop for 5 min. Plates were read at 405 nm.

PCR amplification and cloning of PCR products. The *acpA* gene homologues were amplified from *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 chromosomal DNA via PCR. The oligodeoxyribonucleotide primers used were AP-5′ (GCTCTAGACGAGCGGACGGGAAATGGCG) contain-

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
E. coli		
DH5α	F⁻ φ80d*lacZ* ΔM15 Δ(*lacZYA–argF*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Bethesda Research Laboratories
Top 10	F⁻ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) φ80*lacZ* ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara–leu*)7697 *galU galK rpsL* (Sm^R) *endA1 nupG*	Invitrogen
SM10 λ*pir*	SM10 with a λ prophage carrying the gene	Miller & Mekalanos (1988)
B. pseudomallei		
1026b	Clinical isolate (human); Km^R Gm^R Pm^R Sm^R Tc^S	DeShazer *et al.* (1997)
DD503	1026b derivative; allelic exchange strain; Δ(*amrR–oprA*); Km^S Gm^S Sm^S *rpsL* Sm^R	Moore *et al.* (1999)
APM402	1026b derivative; ORF::Tn*5*-OT182	This study
APM403	1026b derivative; *apcA*::Tn*5*-OT182	This study
APM403C	APM403 (p29*acpA*)	This study
MB401Z	DD503 derivative; *acpA*Δ(nt 1–1139)::*shble*-p15Aori; Ze^R	This study
MB401	DD503 derivative; *acpA* Δ(nt 1–1139)	This study
B. thailandensis		
E264	Environmental isolate; Km^R Gm^R Pm^R Sm^R Tc^S	Brett *et al.* (1998)
DW503	E264 derivative; allelic exchange strain; Δ(*amrR–oprA*); Km^S Gm^S Sm^S; *rpsL* Sm^R	This study
APM501	E264 derivative; *udp–rfaH*::Tn*5*-OT182	This study
APM502	E264 derivative; *apcA*::Tn*5*-OT182	This study
APM501C	APM501 (p29*acpA*)	This study
DW401Z	DW503 derivative; *acpA*Δ(nt 1–1139)::*shble*-p15Aori; Ze^R	This study
DW401	DW503 derivative; *acpA* Δ(nt 1–1139)	This study
B. mallei		
ATCC 23344 (formerly GB8)	Clinical isolate (equine)	USAMRIID
G8N	ATCC 23344 derivative; Nx^R	This study
G8P	ATCC 23344 derivative; *acpA*Δ(nt 1–1139)::*shble*-p15oriV; Ze^R	This study
G8PN	G8P derivative; Ze^R Nx^R	This study
Plasmids		
pOT182	pSUP102(Gm)::Tn*5*-OT182; Cm^R Gm^R Ap^R Tc^R	Merriman & Lamont (1993)
pUC19	Cloning vector	New England Biolabs
pCR2.1TOPO	Cloning vector; pUC ori; Ap^R Km^R	Invitrogen
pUCP29T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp^R	Schweizer *et al.* (1996)
p34EoriZeo	Vector containing self-cloning Ze^R cassette; *shble*-p15oriV; this vector was derived from p34Eori and the Zeo cassette from pEM7/Zeo (Invitrogen)	P. J. Brett, D. DeShazer, M. N. Burtnick & D. E. Woods, unpublished
pKAS46	Allelic exchange vector; *rpsL* Sm^S	Skorupski & Taylor (1996)
pAPM402E	0·7 kb *Eco*RI fragment from APM402 obtained by self-cloning; Ap^R Tc^R	This study
pAPM403E	8·0 kb *Eco*RI fragment from APM403 obtained by self-cloning; Ap^R Tc^R	This study
pAPM403H	5·5 kb *Hin*dIII fragment from APM403 obtained by self-cloning; Ap^R Tc^R	This study
pAPM501Ss	1·6 kb *Sst*I fragment from APM501 obtained by self-cloning; Ap^R Tc^R	This study
pAPM501H	6·5 kb *Hin*dIII fragment from APM501 obtained by self-cloning; Ap^R Tc^R	This study
pAPM502E	0·5 kb *Eco*RI fragment from APM502 obtained by self-cloning; Ap^R Tc^R	This study
pMB401	pUC19 containing 4·5 kb *Hpa*I/*Sst*I fragment containing nt 1–1633 of the *acpA* gene	This study
pMB401X	pMB401 Δ(2·8 kb *Xho*I fragment)	This study
pMB401Z	pMB401 Δ(2·8 kb *Xho*I fragment); *shble*-p15AoriV; Ze^R	This study
p46MB401X	pKAS46 containing 1·3 kb *Sst*I/*Hin*dIII fragment from pMB401X	This study
p46MB401Z	pKAS46 containing 2·7 kb *Sst*I/*Hin*dIII fragment from pMB401Z; Ze^R	This study
p29*acpA*	pUCP29T with 1·8 kb *Xba*I/KpnI fragment containing *B. pseudomallei acpA* gene	This study
pRT733	*oriR6K mobRP4* Tn*phoA*; Ap^R Km^R	Taylor *et al.* (1989)
pmini-O*phoA*	pMB1 *oriR*, Tn*5* *tnp*, RP4 *oriT*, *phoA*; Gm^R	Bolton & Woods (2000)
pBR322	Cloning vector oriColE1; Ap^R Tc^R	New England Biolabs

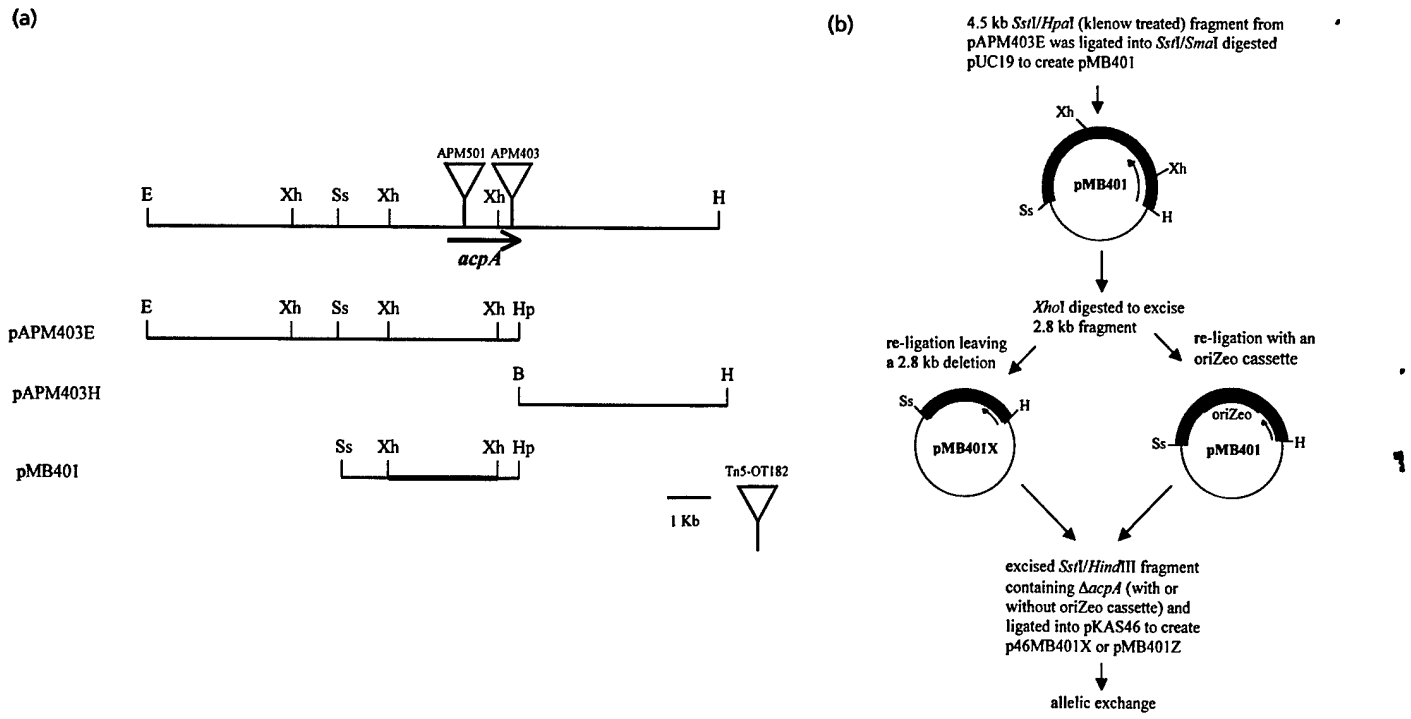


Fig. 1. (a) Plasmids containing *acpA* genes used in this study. Relative locations of Tn*5*-OT182 integrations in the *acpA* gene (black arrow) in AP-negative mutants, APM403 and APM501, are shown. Fragments obtained by self-cloning are shown, pAPM403E and pAPM403H. The fragment from pMB401 used for construction of strains for use in Tn*phoA* mutagenesis is also shown; the 2·8 kb fragment deleted encompassing nucleotides 1–1139 of the *acpA* gene is shown as a thicker black line. (b) Scheme used for deletion of a portion of the *B. pseudomallei acpA* gene (black arrow on inside of plasmid) and subsequent construction of vectors for allelic exchange. Restriction sites are as follows: *Eco*RI (E), *Hind*III (H), *Xho*I (Xh), *Sst*I (Ss), *Hpa*I (Hp) and *Bam*HI (B). The Hp and B sites were located on the ends of Tn*5*-OT182.

ing an *Xba*I linker and AP-3′ (GGGGTACCTCTTGT-CTACCGTACCGACC) containing a *Kpn*I linker (linkers underlined). PCR amplification was performed in 100 µl reaction mixtures containing approximately 500 ng genomic DNA, 1 × PCR buffer (GibcoBRL), a 200 mM concentration of each deoxynucleoside triphosphate, a 0·5 mM concentration of each primer, 2 mM $MgCl_2$ (GibcoBRL), 1 × Q-solution (Qiagen) and 5 U *Taq* DNA polymerase (GibcoBRL) per µl. This mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycler and subjected to a 5 min denaturation step at 95 °C followed by 30 cycles at 95 °C for 45 s, 56 °C for 30 s and 72 °C for 90 s. The reaction mixture was next held at 72 °C for 10 min and then placed at 4 °C until analysed on a 1% agarose gel. The resulting PCR products were digested with *Kpn*I and *Xba*I and cloned into pUC19 or cloned directly into pCR2.1TOPO (Invitrogen) using a TOPO TA Cloning Kit (Invitrogen). The cloned PCR products were sequenced on both strands.

Construction of allelic exchange mutants. Allelic exchange was performed in *B. pseudomallei* DD503 and *B. thailandensis* DW503 using the *rpsL*-based vector pKAS46 as previously described (Moore *et al.*, 1999; Skorupski & Taylor, 1996). Both DD503 and DW503 are Sm^S due to deletion of the *amrR–oprA* operon, but Sm^R due to a mutation in the *rpsL* gene (Moore *et al.*, 1999). Allelic exchange experiments in the present study employed the vectors p46MB401Z or p46MB401X, which were constructed using a deleted version of the *acpA* gene homologue from *B. pseudomallei*. The steps in construction of these vectors are described below and are also shown in Fig. 1(b). A 4·5 kb *Sst*I/*Hpa*I (Klenow-treated) fragment containing nucleotides 1 to 1633 of the *acpA* gene was excised from pAPM403E and inserted into pUC19, creating pMB401. A 2·8 kb *Xho*I fragment was then excised from pMB401 and the vector was ligated back together with or without an oriZeo cassette, resulting in pMB401X or pMB401Z. Each of these fragments containing the Δ*acpA* gene homologue was separately inserted into pKAS46 to create p46MB401Z or p46MB401X. SM10 *λpir* strains containing these vectors were used in conjugation experiments with either DD503 or DW503. Transconjugants were selected for on LB agar containing 50 µg Pm ml^{-1}, 50 µg Km ml^{-1} and 100 µg Ze ml^{-1} when appropriate. Transconjugants were subsequently plated on 100 µg Sm ml^{-1} alone or with 100 µg Ze ml^{-1} to select for loss of the vector. These mutants were plated on LB agar plates containing 50 µg Km ml^{-1} to confirm a double crossover event as indicated by lack of growth. Allelic exchange mutants were confirmed by Southern blot analysis.

A slightly different strategy was used for allelic exchange in *B. mallei*, because spontaneous Sm^R mutants of ATCC 23344 were also Sm dependent, i.e. these mutants could not grow in the absence of Sm. To overcome this problem, transconjugants from ATCC 23344 and SM10 *λpir*(p46MB401Z) mutagenesis were selected for on TSG agar containing 80 µg XP ml^{-1}, 15 µg Pm ml^{-1} and 5 µg Ze ml^{-1}. One white transconjugant was Km^S, indicating loss of the vector (pKAS46) and thus, a double crossover event. This mutant was designated G8P. A spontaneously Nx^R derivative of G8P was selected for and obtained because Nx provided better selection than Pm. This Nx^R Ze^R XP-negative strain of *B. mallei* was designated G8PN and was used for further assessment.

Complementation of Tn*5*-OT182 mutants. A wild-type copy of the *acpA* gene obtained by PCR from *B. pseudomallei* 1026b DNA was cloned into the broad-host-range vector pUCP29T. This construct, designated p29*acpA*, was transformed into *E. coli* SM10 *λpir* and conjugated to *B. pseudomallei* APM403 and *B. thailandensis* APM501 for 5 h, followed by selection on LB agar plates containing 100 μg Sm ml^{-1} and 100 μg Tp ml^{-1}. The resulting strains were inoculated on similar LB agar plates with 40 μg XP ml^{-1} and blue colonies were retained.

Tn*phoA* and mini-O*phoA* mutagenesis. In a typical Tn*phoA* mutagenesis experiment, approximately 5 μl of an overnight culture of SM10 *λpir*(pRT733) containing Tn*phoA* and 5 μl of either *B. pseudomallei* MB401Z or *B. thailandensis* DW401Z were mixed together on an LB agar plate and incubated at 37 °C for 18 h. Eight to ten separate conjugations were carried out on a single plate concurrently along with donor and recipient alone as controls. Each individual conjugation was plated on a single agar plate. *B. pseudomallei* and *B. thailandensis* transconjugants were selected for on LB agar containing 300 μg Km ml^{-1}, 100 μg Sm ml^{-1}, 100 μg Ze ml^{-1} and 40 μg XP ml^{-1}. For *B. mallei* G8PN a similar procedure was employed except that transconjugants were selected for on TSG agar plates containing 5 μg Km ml^{-1}, 75 μg Nx ml^{-1}, 5 μg Ze ml^{-1} and 80 μg XP ml^{-1}. Plates were incubated at 37 °C for 48 h and blue colonies were retained for further analysis. The DNA immediately flanking the Tn*phoA* integration was cloned as previously described (Taylor *et al.*, 1989) using the cloning vector pBR322 and *Bam*HI- or *Sal*I-digested genomic DNA. The resulting plasmids were sequenced using a previously described primer sequence (Taylor *et al.*, 1989)

Mini-O*phoA* was constructed using the Tn*5*-based plasposon pTnModOGm (Dennis & Zylstra, 1998) and the *phoA* gene from pRT733 (Tn*phoA*) (Manoil & Beckwith, 1985). Mini-O*phoA* is small (3·4 kb) and contains an origin of replication that allows for self-cloning of the chromosomal DNA adjacent to transposon integrations (Bolton & Woods, 2000). *B. pseudomallei* MB401, *B. thailandensis* DW401 and *B. mallei* G8PN strains were recipient strains for mini-O*phoA* mutagenesis experiments. Conjugations were performed as described for Tn*phoA* using 5 μl of donor and recipient strains on LB or TSG agar plates at 37 °C for 18 h. Transconjugants of *B. pseudomallei* and *B. thailandensis* were selected for on LB agar plates containing 100 μg Sm ml^{-1}, 15 μg Gm ml^{-1} and 40 μg XP ml^{-1}. *B. mallei* transconjugants were selected for on TSG agar plates containing 5 μg Gm ml^{-1}, 75 μg Nx ml^{-1}, 5 μg Ze ml^{-1} and 80 μg XP ml^{-1}. Self-cloning of the DNA immediately flanking mini-O*phoA* integrations was performed essentially as previously described for Tn*5*-OT182 (DeShazer *et al.*, 1997). Briefly, genomic DNA of mutants harbouring mini-O*phoA* was isolated then digested with *Not*I at 37 °C for 1 h. These reactions were then heat-inactivated followed by ethanol precipitation. Ligation reactions were set up for 1 h at room temperature or overnight at 16 °C then transformed into chemically competent *E. coli* DH5α or Top 10 cells. The resulting plasmids were isolated and sequenced.

DNA sequencing and analysis. DNA sequencing was performed by University Core DNA Services (University of Calgary). The previously described oligodeoxyribonucleotide primers OT182-RT and OT182-LT (DeShazer *et al.*, 1997) were used for sequencing of plasmid DNA obtained by self-cloning of Tn*5*-OT182 mutants. The previously described primer sequence (5′-AATATCGCCCTGAGC-3′) was used for sequencing plasmids from Tn*phoA* clones obtained in this study (Taylor *et al.*, 1989). Two deoxyoligonucleotide primers, Pho-LT (5′-CAGTAATATCGCCCTGAGCAGC-3′) and Gm-RT (5′-GCCGCGCAATTCGAGCTC-3′), were used for sequencing the mini-O*phoA* clones (Bolton & Woods, 2000). Custom-designed primers were synthesized by University Core DNA Services and used in a primer walking strategy to obtain the sequence of both strands of the *acpA* gene homologue.

The DNA sequences obtained in this study were analysed using DNASIS v2.5 (Hitachi) and DIALIGN 2.1 (Morgenstern, 1999) for the presence of ORFs and restriction endonuclease cleavage sites, for sequence alignment and for translation to amino acid sequences. BLASTX and BLASTP programs were used to perform database searches in order to establish homology to known gene sequences (Altschul *et al.*, 1997).

The *acpA* gene sequences from *B. pseudomallei*, *B. thailandensis* and *B. mallei* were submitted to GenBank under accession nos AF252862, AF252863 and AF276770, respectively.

RESULTS

Identification of *acpA* homologues from *B. pseudomallei*, *B. thailandensis* and *B. mallei*

To identify and characterize the gene or genes responsible for the phosphatase activity exhibited by *B. pseudomallei*, we chose to employ Tn*5*-OT182 mutagenesis in combination with a simple screen in which the chromogenic substrate XP was incorporated into LB agar plates. Approximately 7000 *B. pseudomallei* Tn*5*-OT182 mutants were plated onto media containing XP. Two mutants were identified that were unable to hydrolyse XP as indicated by their lack of blue colour. These two *B. pseudomallei* phosphatase-negative mutants were designated APM402 and APM403. The DNA flanking the Tn*5*-OT182 integrations in each of these mutants was isolated by self-cloning using *Eco*RI and *Hin*dIII. The resulting plasmids were isolated and single-stranded sequence reactions were carried out. The sequences obtained were analysed using the BLASTX local alignment search tool. The sequence from APM403 demonstrated a high degree of homology to the AP of *Francisella tularensis* var. *novicida*. In contrast, the sequence from APM402 showed no significant homology to any sequences currently in the GenBank database.

The self-cloned plasmids, pAPM403E and pAPM403H (Fig. 1a), were sequenced for approximately 2 kb on each side of the Tn*5*-OT182 integration on both strands using a primer walking strategy. An ORF of 1734 nucleotides was identified. The product of this ORF demonstrated 36% similarity to the *acpA* gene of *F. tularensis* and was therefore designated the *B. pseudomallei acpA* gene homologue. PCR primers were designed based on this sequence in order to identify the *acpA* gene homologues of *B. thailandensis* and *B. mallei*, both of which exhibit phosphatase activity. This approach was successful for identification of the *B. mallei acpA* homologue, but ineffective for identifying the *B. thailandensis acpA* homologue. The PCR product obtained from *B. mallei* ATCC 23344 chromosomal DNA using the AP-5′ and AP-3′ primers migrated to the

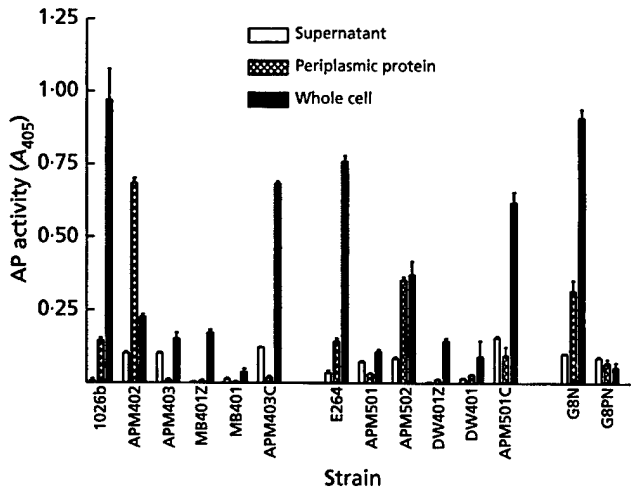


Fig. 2. AP activities of the *B. pseudomallei*, *B. thailandensis* and *B. mallei* strains used in this study. Supernatant, periplasmic and whole-cell fractions were prepared from overnight cultures grown at 37 °C. See Methods for details. The values are means and standard deviations of a single experiment performed in triplicate.

same position as the *B. pseudomallei* 1026b PCR product on a 1% agarose gel; both were approximately 1·8 kb in size. This result indicated that the *acpA* gene homologues present in both *B. pseudomallei* and *B. mallei* were probably very similar. These products were cloned and subjected to sequence analysis, which confirmed them to be *acpA* homologues. The sequence of the *B. mallei acpA* homologue was then completed on both strands.

Since we were unable to obtain a *B. thailandensis acpA* homologue by PCR, we chose to employ Tn5-OT182 mutagenesis to isolate this gene. Approximately 5000 Tn5-OT182 mutants of *B. thailandensis* E264 were plated onto LB agar plates containing XP and two white mutants were obtained. These mutants were designated APM501 and APM502. The chromosomal DNA immediately flanking the Tn5-OT182 integrations in these mutants was obtained by self-cloning using *Sst*I and *Hin*dIII. The sequences obtained from pAPM501Ss and pAPM501H demonstrated highest homology to the *B. pseudomallei acpA* gene homologue. Primer walking was employed to sequence the *B. thailandensis acpA* homologue on both strands. The sequence from the *B. thailandensis* APM502 was shown to have highest homology to the UDP-*rfaH* intergenic region of *E. coli*.

AP activity of *B. pseudomallei*, *B. thailandensis* and *B. mallei* strains

AP activity has previously been characterized for *B. pseudomallei* and it has been shown that optimal activity occurs at pH 5·5 and at 37 °C (Kanai & Kondo, 1994; Kondo *et al.*, 1991a, 1996). To confirm that the mutant strains isolated in this study lacked any AP activity, both the parent strains and the mutant strains were assayed as described in Methods (Fig. 2). All three parent strains, *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. mallei* ATCC 23344, demonstrated similar levels of AP activity. The results of this assay indicated that the Tn5-OT182 mutants, APM403 and APM501, lacked any observable AP activity. In contrast, APM402 had considerable AP activity restricted to the periplasmic fraction and APM502 retained observable amounts of AP activity in both the periplasmic and whole-cell fractions.

Nucleotide sequence analysis of the *acpA* genes of *B. pseudomallei*, *B. thailandensis* and *B. mallei*

The nucleotide sequences of the *acpA* gene homologues from *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. mallei* ATCC 23344 demonstrated 23% identity and 36% similarity with the *acpA* gene product of *F. tularensis*. A 1734 bp ORF was identified, beginning with the ATG codon and ending with the TGA codon, that was consistent for *B. pseudomallei* and *B. mallei*, while a GTG start codon was identified for *B. thailandensis*. The G+C content of the *acpA* ORF was determined to be 69 mol%. A putative Shine–Dalgarno sequence was identified −6 to −10 bp upstream of the putative ATG/GTG start codons, suggesting that these are the correct start sites. The nucleotide sequences obtained for *B. thailandensis* and *B. mallei* were 94% and 99% identical, respectively, to the *B. pseudomallei acpA* sequence. The Tn5-OT182 integration associated with APM403 occurred at nucleotide 1633 of the *acpA* ORF while the Tn5-OT182 integration associated with APM501 occurred at nucleotide 365. The approximate positions are shown in Fig. 1(a). The putative protein encoded by *acpA* was predicted to be 578 amino acids in length with a calculated molecular mass of 62860 Da. Comparison of the predicted amino acid sequences of *B. thailandensis* AcpA and *B. mallei* AcpA to that of the *B. pseudomallei* AcpA predicted amino acid sequence revealed 15 and 3 differences, respectively. This reflects the close phylogenetic relationship between these species.

Characterization of AP-negative allelic exchange mutants

AP-negative mutants were constructed by allelic exchange as previously described with *B. pseudomallei* DD503 and *B. thailandensis* DW503 using the vectors p46MB401Z or p46MB401X (Fig. 1b). These vectors contained a portion of the *acpA* gene including nucleotides 1140–1633 along with 1·5 kb of upstream DNA that has not yet been sequenced. The *B. pseudomallei* Δ*acpA* mutants were designated MB401Z and MB401 and the *B. thailandensis* Δ*acpA* mutants were designated DW401Z and DW401. The strains MB401 and DW401 have a deletion in their *acpA* genes and lack the oriZeo marker present in the other AP-negative allelic exchange mutants, thus eliminating the need for the presence of Ze in selective media. As described in Methods, a slightly different strategy using the p46MB401Z vector was employed for allelic exchange in *B. mallei*. The

oriZeo cassette was used for positive selection in *B. mallei* allelic exchange and Ze was used in further experiments. The resulting Δ*acpA* strain of *B. mallei* was designated G8PN and was assessed for AP activity.

The isogenic allelic exchange mutant strains MB401Z/MB401, DW401Z/DW401 and G8PN were unable to hydrolyse the chromogenic substrate XP when present in LB or TSG agar. This was consistent with the observation that Tn5-OT182 disruptions in the *acpA* homologue caused APM403 and APM501 to display a white phenotype. Additionally, the allelic exchange mutants were essentially devoid of AP activity at pH 5·5 (Fig. 2) compared to wild-type strains. The inability of these strains to display blue colour when grown on agar containing XP made them good candidates as recipients for mutagenesis with Tn*phoA* and thus for the identification of exported products.

Complementation of AP-negative Tn5-OT182 strains

The 1·8 kb PCR product harbouring the *acpA* gene homologue *B. pseudomallei* was cloned into pUCP29T and was conjugated to *B. pseudomallei* APM403 and *B. thailandensis* APM501. The presence of p29*acpA* was able to restore the AP activity of these strains. The complemented strains, designated APM403C and APM501C, were blue when grown on LB agar plates containing XP and exhibited activity by AP activity assay (Fig. 2). These results indicate that the AP-negative phenotype observed in APM403 and APM501 is due to the Tn5-OT182 disruption in their *acpA* gene homologues and that this gene encodes a product that is responsible for the AP activity observed in these organisms. The strains constructed for Tn*phoA* mutagenesis were not complemented as the mutation encompasses 2·8 kb that has not been completely sequenced.

Tn*phoA* and mini-O*phoA* mutagenesis of *B. pseudomallei*, *B. thailandensis* and *B. mallei*

Two Tn5-based transposons containing truncated *phoA* genes were employed in this study. Initially, Tn*phoA* was delivered to MB401Z, DW401Z and G8PN on the vector pRT733 as previously described (Taylor *et al.*, 1989). This system worked efficiently for *B. pseudomallei* and *B. thailandensis*, resulting in approximately 1000–1200 Sm^R Km^R transconjugants per mutagenesis experiment, 1% of which were PhoA positive. However, in *B. mallei*, the Tn*phoA* transposition frequency was significantly lower: each mutagenesis resulted in only 50–200 Nx^R Km^R transconjugants with a frequency of PhoA-positive colonies of approximately 2%. Southern blot analysis using *Bam*HI-digested chromosomal DNA from Tn*phoA* mutants confirmed that Tn*phoA* integrated only once per chromosome in four randomly selected *B. pseudomallei* and *B. thailandensis* PhoA-positive mutants.

Although this system is functional in these strains, the cloning procedures had a low efficiency, approximately 25%. This is suspected to be due in part to the size of the transposon and the fact that the cloning vector, pBR322, has a size limit on the DNA inserts that it can efficiently accept (approx. 7 kb). Upon digestion of the chromosomal DNA of PhoA-positive mutants with *Bam*HI or *Sal*I at least 5 kb of transposon remains along with the chromosomal DNA immediately flanking fragment. The cloning of DNA fragments containing Tn*phoA* and adjacent chromosomal DNA into pBR322 resulted in only relatively small (<2 kb) flanking DNA sequences being obtained. The resulting plasmids were sequenced and BLASTX searches were performed. Sequences showing significant homology over at least 300 bp of flanking DNA are shown in Table 2.

Due to the low numbers of transconjugants in *B. mallei* Tn*phoA* mutagenesis experiments, we chose to employ a second transposon, designated mini-O*phoA* (Bolton & Woods, 2000). The mini-O*phoA* system was found to be equivalent in transposition frequency to Tn*phoA* in *B. pseudomallei* and *B. thailandensis*, with the occurrence of PhoA-positive colonies being 0·5%. The transposition frequency was increased in *B. mallei*: approximately 1500 Ze^R Nx^R Gm^R transconjugants were obtained per mutagenesis, with the frequency of PhoA-positive colonies being 2%. In addition, due to the presence of an origin of replication, the cloning procedures for obtaining the DNA sequences flanking mini-O*phoA* integrations were simpler and more efficient than those for Tn*phoA*. Cloning efficiency was 90% when chemically competent *E. coli* Top 10 cells (Invitrogen) were used. The chromosomal DNA of six random Sm^R Gm^R transconjugants of *B. pseudomallei* and *B. thailandensis* and three random Ze^R Nx^R Gm^R transconjugants of *B. mallei* was isolated, digested with *Not*I and probed with α-^{32}P-labelled mini-O*phoA*. All nine mutants contained single copies of this transposon, suggesting that mini-O*phoA* integrates randomly into the chromosomes of *B. pseudomallei*, *B. thailandensis* and *B. mallei*.

The DNA from a number of PhoA-positive *B. pseudomallei*, *B. thailandensis* and *B. mallei* mini-O*phoA* mutants was self-cloned and subjected to single-stranded sequencing in order to characterize the DNA flanking the transposon integrations. Approximately 500–700 bp of sequence was obtained on each side of the mini-O*phoA* integrations. Subsequently, database searches were performed in order to establish homologies to known gene sequences. Some of the sequences obtained from the Pho-LT primer demonstrated significant homology over at least 300 bp and are shown in Table 2. A number of putative genes were identified which encoded proteins showing homology to secreted proteins, confirming the ability of this system to identify extracytoplasmic products expressed by the three *Burkholderia* spp. utilized in this study.

The DNA sequences adjacent to Tn*phoA* and mini-O*phoA* integrations in a number of PhoA-positive mutants did not show any significant homology to sequences currently in the GenBank database. These

Table 2. Table of homology of Tn*phoA* and mini-O*phoA* flanking sequences

PhoA-positive mutant	Identity (%)	Similarity (%)	Homology	Entrez protein ID
B. pseudomallei **Tn*phoA* mutants**				
PHOA8	40	43	Putative cell wall protein of *Streptomyces coelicolor*	AL137165
PHOA16	99	99	*gspG* (*B. pseudomallei*) type II secretion pathway gene	AAD05177.1
PHOA20	23	33	Hydroxyproline-rich glycoprotein of *Zea diploperennis*	228938
PHOA39	31	38	ExiT protein (exochelin ABC transporter) from *Mycobacterium smegmatis*	AAC32046.1
PHOA47	32	50	Outer-membrane protein C of *Pseudomonas aeruginosa*	BAA05664.1
B. mallei **Tn*phoA* mutant**				
AJB34	75	95	Dipeptide transport system permease protein of *Escherichia coli*	AAC76568.1
B. pseudomallei **mini-O*phoA* mutants**				
PHOG4	33	43	Penicillin-binding protein of *Deinococcus radiodurans*	AAF10059.1
PHOG9	45	63	Periplasmic serine protease from *Aquifex aeolicus*	AAC07399.1
PHOG18	52	76	Putrescine-binding periplasmic protein precursor; permease protein from *Escherichia coli*	AAC73941.1
PHOG28	69	84	Phosphate-binding periplasmic protein precursor of *Erwinia carotovora*	AAB70458.1
PHOG29	37	50	Outer-membrane porin protein OpcP1 of *Burkholderia cepacia*	BAA09892.1
B. thailandensis **mini-O*phoA* mutants**				
PHOG103	36	44	Putative YME1 ATP-dependent zinc protease of *Arabidopsis thaliana*	AAC31223.1
B. mallei **mini-O*phoA* mutant**				
AJB101	43	56	Probable ABC transporter, permease protein of *Treponema pallidum*	F71375
AJB116	40	60	Branched-chain amino acid ABC transporter of *Deinococcus radiodurans*	H75444
AJB139	69	80	Phosphate-binding protein of *Enterobacter cloacae*	BAA22861.1
AJB150	36	50	Putative aromatic efflux pump outer-membrane protein of *Sphingomonas aromaticivorans*	AAD03862.1
AJB153	60	73	Periplasmic sorbitol-binding protein of *Rhodobacter sphaeroides*	AAC45766.1
AJB171	63	75	Outer-membrane protein C of *Pseudomonas aeruginosa*	BAA05664.1

sequences are of significant interest and may represent as yet undefined genes encoding exported products.

DISCUSSION

The AP activity of *B. pseudomallei* has been previously documented (Kanai & Kondo, 1994; Kondo *et al.*, 1991a, b, 1996); however, the gene responsible for this activity had not been identified prior to this study. The present work has demonstrated the presence of *acpA* gene homologues in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. A simple screen was employed to identify mutants devoid of phosphatase activity. Several Tn*5*-OT182 mutants unable to hydrolyse XP were isolated and these subsequently facilitated the identification of *acpA* gene homologues in both *B. pseudomallei* and *B. thailandensis*. PCR primers designed from the nucleotide sequence of the *B. pseudomallei acpA* gene allowed the identification of an *acpA* homologue from *B. mallei*. The *acpA* genes from the species used in this study were sequenced and the predicted amino acid compositions reflected the close phylogenetic relationship between these species. Complementation analyses have shown that functional *acpA* gene homologues are required for AP activity in these three *Burkholderia* spp. Furthermore, identification of *acpA* genes in these species has allowed the construction of strains with Δ*acpA* genes through allelic exchange.

The AP-negative strains constructed in this study have been used for mutagenesis experiments employing Tn*5*-based transposons containing truncated *phoA* genes. The *B. thailandensis* strain DW401/DW401Z will be particularly useful as it is a non-virulent strain that can be used as a laboratory tool for the identification of genes likely to be present in the highly virulent, closely related *B. pseudomallei* and *B. mallei* strains. The results of this study clearly indicate that Tn*5*-based transposons containing truncated *phoA* genes can be efficiently used in *B. pseudomallei* and *B. thailandensis* strains. It is not clear why Tn*phoA* mutagenesis was not effective in *B.*

mallei; it may be due to an incompatibility with the vector carrying the transposon. However, this problem was overcome by employing a second transposon system, mini-O*phoA*, that was shown to integrate efficiently in this species.

PhoA-positive transposon mutants have been isolated in this study and sequence analysis of DNA flanking transposon insertions has revealed homology to a number of known gene sequences. We have demonstrated that the *phoA* fusion approach can be efficiently used in *Burkholderia* spp. for the identification of genes encoding exported proteins. The *phoA* systems employed in this study have facilitated the identification of genes potentially contributing to the pathogenesis of melioidosis and glanders. We are currently constructing isogenic mutants in specific genes identified via *phoA* mutagenesis; this will allow for the assessment of the contribution of particular genes to the phenotypes displayed by these organisms. Such mutants will be used in virulence testing. This will help to establish the roles specific exported products play in pathogenesis.

Preliminary studies on the role of the *acpA* gene product in the pathogenesis of *B. pseudomallei* and *B. mallei* infections indicate that the disruption of the *acpA* gene does not significantly alter virulence (data not shown). The mutants harbouring disrupted *acpA* genes may be useful for future studies regarding the specific functioning of the *acpA* gene and its product. Identification of the *acpA* gene and the subsequent implementation of *phoA* mutagenesis systems described in the present study will contribute to the continuing studies on the pathogenesis of melioidosis and glanders.

ACKNOWLEDGEMENTS

This research is supported by the Canadian Bacterial Diseases Network Centers for Excellence. M. N. B. is the recipient of an Alberta Heritage Foundation for Medical Research (AHFMR) studentship. D. W. is the recipient of an AHFMR summer studentship.

REFERENCES

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Ames, G. F., Prody, C. & Kustu, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol* **160**, 1181–1183.

Arun, S., Neubauer, H., Gurel, A., Ayyildiz, G., Kuscu, B., Yesildere, T., Meyer, H. & Hermanns, W. (1999). Equine glanders in Turkey. *Vet Rec* **144**, 255–258.

Bolton, A. J. & Woods, D. E. (2000). A self-cloning minitransposon *phoA* gene fusion system promotes the rapid analysis of secreted proteins in Gram negative bacteria. *Biotechniques* **29**, 470–474.

Bondre, V. P., Srivastava, R., Sinha, V. B. & Srivastava, B. S. (1997). Screening of TnphoA mutants of *Vibrio cholerae* O139 for identification of antigens involved in colonisation. *J Med Microbiol* **46**, 1007–1011.

Brett, P. J., DeShazer, D. & Woods, D. E. (1998). *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *Int J Syst Bacteriol* **48**, 317–320.

Dennis, J. J. & Zylstra, G. J. (1998). Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl Environ Microbiol* **64**, 2710–2715.

DeShazer, D., Brett, P. J., Carlyon, R. & Woods, D. E. (1997). Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterisation of the flagellin structural gene. *J Bacteriol* **179**, 2116–2125.

DeShazer, D., Brett, P. J., Burtnick, M. N. & Woods, D. E. (1999). Molecular characterisation of genetic loci required for secretion of exoproducts in *Burkholderia pseudomallei*. *J Bacteriol* **181**, 4661–4664.

Hsia, R. C., Small, P. L. & Bavoil, P. M. (1993). Characterisation of virulence genes of enterinvasive *Escherichia coli* by Tn*phoA* mutagenesis: identification of *invX*, a gene required for entry into HEp-2 cells. *J Bacteriol* **175**, 4817–4823.

Kanai, K. & Kondo, E. (1994). Recent advances in biomedical sciences of *Burkholderia pseudomallei* (basonym: *Pseudomonas pseudomallei*). *Jpn J Med Sci Biol* **47**, 1–45.

Kondo, E., Dejsirilert, S., Wejprasit, N., Chiewsilp, D. & Kanai, K. (1991a). Heat-stable and heat-labile components of nonspecific acid phosphatase detected in *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* **44**, 51–62.

Kondo, E., Petkanchanapong, V., Naigowit, P., Kurata, T. & Kanai, K. (1991b). Demonstration of acid phosphatase activity in antigenic glycoprotein fractions obtained from the culture filtrate of *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* **44**, 213–224.

Kondo, E., Kurata, T., Naigowit, P. & Kanai, K. (1996). Evolution of cell-surface acid phosphatase of *Burkholderia pseudomallei*. *Southeast Asian J Trop Med Public Health* **27**, 592–599.

Manoil, C. & Beckwith, J. (1985). Tn*phoA*: a transposon probe for protein export signals. *Proc Natl Acad Sci U S A* **82**, 8129–8133.

Merriman, T. R. & Lamont, I. L. (1993). Construction and use of a self-cloning promoter probe vector for gram-negative bacteria. *Gene* **126**, 17–23.

Miller, V. L. & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**, 2573–2583.

Milner, J. S., Dymock, D., Cooper, R. M. & Roberts, I. S. (1993). Penicillin-binding proteins from *Erwinia amylovora*: mutants lacking PBP2 are avirulent. *J Bacteriol* **175**, 6082–6088.

Moore, R. A., DeShazer, D., Reckseidler, S. L., Weissman, A. & Woods, D. E. (1999). Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* **43**, 465–470.

Morgenstern, B. (1999). DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. *Bioinformatics* **15**, 211–218.

Reckseidler, S. L., DeShazer, D., Sokol, P. A. & Woods, D. E. (2001). Detection of bacterial virulence genes by subtractive hybridisation: identification of the capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. *Infect Immun* **69** (in press).

Rubino, S., Leori, G., Rizzu, P., Erre, G., Colombo, M. M., Uzzau, S., Masala, G. & Cappuccinelli, P. (1993). Tn*phoA* *Salmonella abortusovis* mutants unable to adhere to epithelial cells with reduced virulence in mice. *Infect Immun* **61**, 1786–1792.

Schweizer, H., Klassen, T. & Hoang, T. (1996). Improved methods for gene analysis and expression in *Pseudomonas* spp. In

Molecular Biology of Pseudomonads, pp. 229–237. Edited by T. Nakazawa, K. Furukawa, D. Haas & S. Silver. Washington, DC: American Society for Microbiology.

Skorupski, K. & Taylor, R. K. (1996). Positive selection vectors for allelic exchange. *Gene* **169**, 47–52.

Smith, C., Allen, J., Embi, M., Othman, O., Razak, N. & Ismail, G. (1987). Human melioidosis: an emerging medical problem. *MIRCEN J Appl Microbiol Biotechnol* **3**, 343–366.

Taylor, R. K., Manoil, C. & Mekalanos, J. J. (1989). Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. *J Bacteriol* **171**, 1870–1878.

Weiser, J. N. & Gotschlich, E. C. (1991). Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *Infect Immun* **59**, 2252–2258.

Received 27 June 2000; revised 12 September 2000; accepted 5 October 2000.

Article available online at http://www.idealibrary.com on IDEAL®

Microbial Pathogenesis 2001; **30**: 253–269
doi:10.1006/mpat.2000.0430

MICROBIAL PATHOGENESIS

Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant

David DeShazer[a]*, David M. Waag[a], David L. Fritz[a] & Donald E. Woods[b]

[a] *U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, U.S.A. and* [b] *University of Calgary, Alberta, Canada*

(Received September 18, 2000; accepted in revised form December 13, 2000)

Little is known about the virulence factors of *Burkholderia mallei*, the etiologic agent of glanders. We employed subtractive hybridization to identify genetic determinants present in *B. mallei* but not in *Burkholderia thailandensis*, a non-pathogenic soil microbe. Three subtractive hybridization products were mapped to a genetic locus encoding proteins involved in the biosynthesis, export and translocation of a capsular polysaccharide. We identified an insertion sequence (IS*407*A) at one end of the capsule gene cluster and demonstrated that it was functional in *B. mallei*. Mutations were introduced in the *B. mallei* capsular gene cluster and the corresponding mutants were examined for their reactivity with antibodies raised against *Burkholderia pseudomallei* surface polysaccharides by immunoblotting and ELISA. Immunogold electron microscopy demonstrated the presence of a capsule on the surface of *B. mallei* ATCC 23344 (parental strain) but not on *B. mallei* DD3008 (capsule mutant) or *B. thailandensis*. Surprisingly, *B. thailandensis* also harboured a portion of the capsule gene cluster. ATCC 23344 was highly virulent in hamsters and mice, but DD3008 was avirulent in both animal models. The results presented here demonstrate that the capsular polysaccharide of *B. mallei* is required for production of disease in two animal models of glanders infection and is a major virulence factor.

Key words: glanders, melioidosis, pathogenesis and animal model.

Introduction

Glanders, one of the oldest diseases known to man, was first described by Aristotle [1]. It is a disease of solipeds (horses, mules and donkeys) but incidental infections also occur in humans and carnivores [2–4]. Glanders was distributed worldwide until control measures were introduced in the early 20th century [5]. The disease has been eradicated from North America and western Europe but still persists in some

* Author for correspondence.
E-mail: david.deshazer@amedd.army.mil

0882–4010/01/050253+17 $35.00/0

South American, eastern European, African and Asian countries. Glanders in humans is almost always fatal without antibiotic intervention and it typically occurs in people who have occupations that put them in contact with glanderous animals such as veterinarians, stablemen and farmers [3, 4, 6]. Laboratory-acquired glanders infections in researchers studying *Burkholderia mallei*, the etiologic agent of glanders, have also been reported [7, 8]. *B. mallei* is a critical biological agent (category B) because of its aerosol infectivity, severe course of infection and worldwide availability [4, 9, 10]. There are concerns that critical biological agents will be used in future acts of biological terrorism [9]. In fact, *B. mallei* was allegedly used as a biological weapon during World War I [11, 12]. No human or veterinary glanders vaccine is currently available.

B. mallei is an obligate parasite of horses, mules and donkeys and no other natural reservoir is known [4, 13]. The organism is a nonmotile Gram-negative bacillus that is closely related to *Burkholderia pseudomallei* and *Burkholderia thailandensis* [14, 15]. *B. pseudomallei* is a pathogenic microbe that causes the glanders-like disease melioidosis [16] and *B. thailandensis* is a nonpathogenic soil saprophyte [14]. Little is known about the molecular biology of the putative virulence determinants of *B. mallei*, as it is poorly characterized genetically. As a result, very little is known about the basic mechanism(s) of *B. mallei* pathogenesis. We are interested in identifying and characterizing those factors that are responsible for *B. mallei* pathogenesis at the molecular level. In this study, we performed subtractive hybridization between *B. mallei* and *B. thailandensis* and identified a genetic locus encoding a major *B. mallei* virulence determinant.

Results

Identification of a *B. mallei* polysaccharide gene cluster by subtractive hybridization

The goal of this study was to identify genetic determinants present in the pathogen *B. mallei*, but not in the non-pathogen *B. thailandensis* via subtractive hybridization. We sequenced 23 distinct plasmid inserts from a *B. mallei–B. thailandensis* subtractive hybridization library and selected three plasmids; pDD3006, pDD3008 and pDD3023 for further study. These plasmids were selected because they contained inserts with relatively low G+C contents as compared to the rest of the *B. mallei* ATCC 23344 genome (68% G+C) [15]. The plasmids pDD3006, pDD3008 and pDD3023 contained DNA inserts with G+C contents of 50, 53 and 57%, respectively. The plasmid inserts were mapped to a genetic locus containing 28 genes, an insertion sequence and a gene remnant [Fig. 1(a)]. The majority of encoded proteins are similar to bacterial proteins involved in capsular polysaccharide biosynthesis, export and translocation [Table 1, Fig. 1(a)]. Genes involved in the biosynthesis of bacterial surface polysaccharides are commonly arranged in clusters and the genes in the cluster reported here have been assigned names based on the bacterial polysaccharide gene nomenclature (BPGN) scheme [17].

Molecular characterization of the *B. mallei* polysaccharide gene cluster

Bacterial polysaccharide biosynthetic gene clusters commonly encode products involved in three distinct processes: (1) biosynthesis of nucleotide sugars, or other components, needed for polysaccharide synthesis; (2) transfer of activated sugars from their nucleotide carriers to a growing carbohydrate chain; and (3) export and translocation of the resulting polysaccharide [17, 18]. The *B. mallei* polysaccharide gene cluster encodes at least 12 proteins involved in biosynthesis of nucleotide sugars, or other components, needed for polysaccharide synthesis [Table 1, Fig. 1(a)]. Included in this group is a protein that is homologous to eukaryotic heparan sulfate 6-sulfotransferases (WcbF) and four proteins that are similar to proteins involved in lipid biosynthesis, modification and transfer (WcbP, WcbR, WcbS and WcbT). WcbB, WcbE and WcbH are homologous to glycosyltransferases, proteins involved in the transfer of activated sugars from their nucleotide carriers to a growing carbohydrate chain [Table 1, Fig. 1(a)]. The proteins WcbA, WcbC, WcbD, Wzm, Wzt and WcbO are similar to proteins involved in export and translocation of group 3 capsular polysaccharides in *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Campylobacter jejuni* and *Pasteurella multocida* [19–22]. Finally, there are two novel proteins (WcbG and WcbI) and four proteins that are homologous to proteins with unknown functions (DedA, YggB, YafJ and WcbQ) [Table 1, Fig.

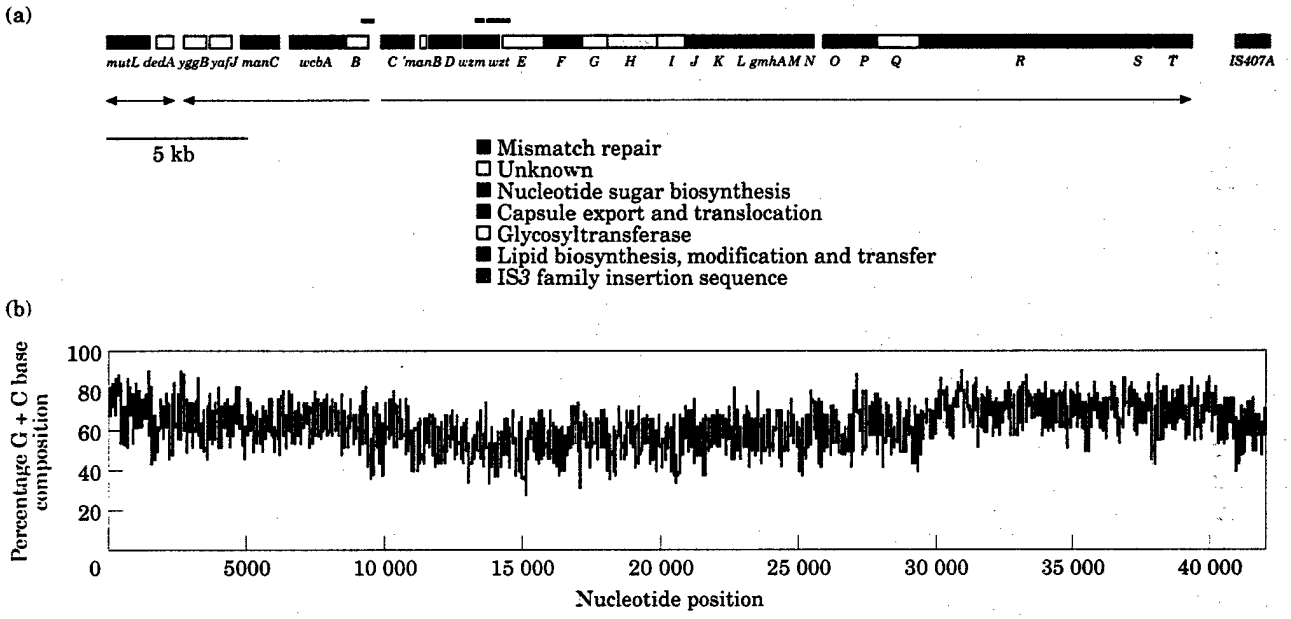


Figure 1. Genetic map and corresponding percentage G+C composition of the *B. mallei* polysaccharide gene cluster. (a) Genetic map of the polysaccharide gene cluster identified using three *B. mallei* subtractive hybridization products. The thin lines above the boxes represent the *B. mallei* subtractive hybridization products from pDD3006, pDD3008 and pDD3023 (Table 3). The boxes represent genes and an insertion sequence, and the arrows represent the transcriptional orientation of genes. The putative functions of the gene products are indicated. A scale bar, representing 5 kb, is shown. (b) The corresponding percentage G+C composition of the genetic locus shown in (a).

1(a)]. Taken together, these results suggest that the *B. mallei* polysaccharide gene cluster may be involved in the biosynthesis of a capsular polysaccharide.

We identified an insertion sequence of the IS3 family [23] 1.6 kb downstream of *wcbT* [Fig. 1(a)]. This IS element was 97% identical (1205 bp/1236 bp) to IS*407* from *Burkholderia cepacia* [24] and was designated IS*407*A. IS*407* and IS*407*A are 1236 bp in length and have 49 bp terminal inverted repeats. Like other members of the IS3 family, IS*407*A contains two partially overlapping reading frames, *orfA* and *orfB*, in the reading phases 0 and −1, respectively [23]. The functional transposase (OrfAB) of the IS3 family is generated by a programmed translational frameshifting event. A similar IS element was recently described in *B. pseudomallei*, but only a partial nucleotide sequence of that element was described [25, 26].

The 480 bp *wcbC–wcbD* intergenic region contains a 216 bp gene remnant that we have designated *'manB* [Fig. 1(a)]. The gene remnant encodes a protein of 72 amino acids with similarity to the carboxy terminal portion of bacterial phosphomannomutases (Table 1). The *manB* and *manC* genes are involved in the GDP–mannose biosynthesis pathway and often occurs in tandem [17]. It should be noted that the *B. mallei* polysaccharide gene cluster contains a copy of *manC* [Fig. 1(a)]. *'manB* is non-functional as it does not contain start or stop codons and may represent the location of a previous genomic rearrangement event involving recombination, deletion or integration.

There is a decrease in the G+C content of the *B. mallei* polysaccharide gene cluster between nucleotide positions 11 000 and 30 000 as compared to the rest of the *B. mallei* genome (68% G+C) [Fig. 1(b)]. The G+C content of the region spanning nucleotide position 11 000 to 30 000 is 58%. In comparison, the G+C content between nucleotide positions 1–11 000 and 30 000–42 146 is 65 and 69%, respectively. Nucleotide sequences acquired via lateral gene transfer often contain a G+C content that differs from that of the rest of the recipient genome [27]. It is interesting to note that the start of the lowered G+C content at nucleotide position 11 000 corresponds to the location of the gene remnant *'manB* (Fig. 1).

Table 1. Predicted *B. mallei* gene products presented in this study and similar proteins in the non-redundant sequence database

Protein	Size (aa/kDa)	Similar protein-Organism	Expect (E) value[a]	Putative function	Database accession number
MutL	—	MutL-*Neisseria meningitidis*	4e-90	Mismatch repair	AE002493
		MutL-*Salmonella typhimurium*	5e-69	Mismatch repair	A33588
DedA	226/24.8	DedA-*E. coli*	2e-57	Unknown	M68935
		DedA-*N. meningitidis*	3e-49	Unknown	AE002455
YggB	290/30.8	YggB-*Edwardsiella ictaluri*	4e-35	Unknown	AF037440
		YggB-*E. coli*	8e-28	Unknown	U28377
YafJ	278/31.5	Yn1191-*Candida albicans*	1e-32	Unknown	AJ250310
		Yn1191wp-*Saccharomyces cerevisiae*	2e-30	Unknown	Z71467
ManC	475/52.2	XanB-*Xanthomonas campestris*	1e-155	GDP-mannose pyrophosphorylase	M83231
		ManC-*E. coli*	1e-147	GDP-mannose pyrophosphorylase	U38473
WcbA	671/74.6	KpsC-*E. coli*	8e-89	Phospholipid substitution	X74567
		LipA-*N. meningitidis*	2e-81	Phospholipid substitution	AE002367
WcbB	280/31.7	WbpX-*Pseudomonas aeruginosa*	3e-29	Glycosyltransferase	AF010181
		MtfA-*Aquifex aeolicus*	8e-22	Mannosyltransferase A	AE000723
WcbC	387/40.9	CpxD-*Actinobacillus pleuropneumoniae*	4e-79	Capsule polysaccharide export	U36397
		CtrA-*N. meningitidis*	2e-78	Capsule polysaccharide export	AE002366
'ManB	72/8.2	ManB-*Yersinia pseudotuberculosis*	0.001	Phosphomannomutase	AJ251712
		manB-*Yersinia pestis*	0.001	Phosphomannomutase	AJ251713
WcbD	382/42.3	BexC-*Haemophilus influenzae*	4e-85	Capsule polysaccharide export	X54987
		CtrB-*N. meningitidis*	2e-78	Capsule polysaccharide export	M57677
Wzm	260/29.2	BexB-*H. influenzae*	3e-62	Capsule polysaccharide export	M33788
		CtrC-*N. meningitidis*	7e-62	Capsule polysaccharide export	M57677
Wzt	218/24.6	BexA-*H. influenzae*	3e-72	Capsule polysaccharide export	M19995
		CpxA-*A. pleuropneumoniae*	3e-71	Capsule polysaccharide export	AF143906
WcbE	507/56.8	MtfB-*A. aeolicus*	3e-19	Mannosyltransferase B	AE000693
		WbpX-*P. aeruginosa*	6e-14	Glycoslytransferase	AF010181
WcbF	440/50.2	BAA89249.1-*Mus musculus*	0.049	Heparan sulfate 6-sulfotransferase 3	AB024567
		BAA89248.1-*M. musculus*	0.11	Heparan sulfate 6-sulfotransferase 1	AB024566
WcbG	313/33.9	None			
WcbH	598/67.4	MtfA-*Archaeoglobus fulgidus*	4e-11	Mannosyltransferase A	AE001103
		SC6G10.05c-*Streptomyces coelicolor*	5e-04	Glycosyltransferase	AL049497
WcbI	311/35.9	None			
WcbJ	280/30.8	F6N23.17-*Arabidopsis thaliana*	1e-10	dTDP-6-deoxy-L-mannose-dehydrogenase	AF058919
		AAB86258.1-*Methanobacterium thermoautotrophicum*	2e-10	dTDP-4-dehydrorhamnose reductase	AE000933
WcbK	337/38.0	Gca-*Mycobacterium tuberculosis*	1e-48	Dehydrogenase	AL021926
		Gmd-*Yersinia pseudotuberculosis*	9e-37	GDP-mannose-4,6-dehydratase	AJ251712
WcbL	346/38.0	Cj1425c-*Campylobacter jejuni*	2e-85	Sugar kinase	AL139078
		Rv0115-*M. tuberculosis*	3e-81	Unknown	AL021926
GmhA	197/20.7	GmhA2-*C. jejuni*	1e-57	Phosphoheptose isomerase	AL139078
		GmhA-*C. jejuni*	3e-46	Phosphoheptose isomerase	AL139077

continued

Table 1. *continued*

Protein	Size (aa/kDa)	Similar protein-Organism	Expect (E) value[a]	Putative function	Database accession number
WcbM	230/24.9	Cj1423c-*C. jejuni*	4e-39	Sugar-phosphate nucleotidyltransferase	AL139078
		AAB86225.1-*M. thermoautotrophicum*	5e-23	mannose-1-phosphate guanyltransferase	AE000931
WcbN	189/20.3	YaeD-*E. coli*	4e-34	Unknown	U70214
		Cj1152c-*C. jejuni*	7e-34	Phosphatase	AL139077
WcbO	400/44.9	KpsS-*E. coli*	2e-62	Phospholipid substitution	X74567
		LipB-*N. meningitidis*	6e-47	Phospholipid substitution	Q05014
WcbP	262/27.8	YurA-*Myxococcus xanthus*	3e-21	Oxidoreductase	P25970
		HetN-*Anabaena* sp. PCC7120	3e-18	Ketoacyl reductase	L22883
WcbQ	511/56.2	RkpI-*Sinorhizobium meliloti*	4e-26	Unknown	Q52938
		Orf7-*Streptococcus mutans*	4e-15	Unknown	AB010970
WcbR	2546/ 267.6	LovF-*Aspergillus terreus*	1e-180	Type I polyketide synthase	AF141925
		EPOS C-*Sorangium cellulosum*	1e-180	Type I polyketide synthase	AF210843
WcbS	305/33.1	LpxC-*H. influenzae*	3e-36	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	U32794
		LpxC-*E. coli*	1e-34	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	X55034
WcbT	439/47.4	RkpG-*S. meliloti*	1e-105	Acyl-CoA transferase	X64131
		Kbl-*E. coli*	1e-57	2-amino-3-ketobutyrate coenzyme A ligase	X06690

[a] A parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.

Mutations in the polysaccharide gene cluster result in capsule-deficient mutants

Little is known about the genetics of *B. mallei*, and there are no published methods for constructing *B. mallei* mutants. In this study, we utilized a mobilizable suicide vector to construct merodiploid strains containing plasmid disruptions of genes in the polysaccharide gene cluster (see Experimental procedures). Since *B. mallei*, *B. pseudomallei* and *B. thailandensis* are closely related serologically [28–32], wild type and mutant strains were examined for their reactivity with rabbit antiserum directed against a *B. pseudomallei* surface polysaccharide with the structure -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1-. Recent studies suggest that this structure represents a capsular polysaccharide rather than a glycoprotein [33, 34] or a LPS O-polysaccharide (O-PS) [35, 36]. *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 demonstrated strong reactivity with the capsular antibodies in an ELISA, but *B. thailandensis* E264 demonstrated only background reactivity (Fig. 2). Seven *B. mallei* mutants (DD3105, DD3008, DD3101, DD3102, DD3103, DD3104 and DD3100) exhibited weak or partial reactivity with the capsular antibodies (Fig. 2). There was no difference in the reactivity of the mutants DD3108 and DD3107 and the parental strain ATCC 23344 in an ELISA (Fig. 2). The results demonstrate that *B. mallei* and *B. pseudomallei* produce an antigenically related polysaccharide capsule that is not present on *B. thailandensis*. The results also suggest that *manC*, *wcbB*, *wcbL*, *wcbM*, *wcbP*, *wcbQ* and *wcbR* are involved in the biosynthesis, export or translocation of the capsule in *B. mallei*. As the genes *yggB* and *yafJ* do not appear to be required for capsule production in *B. mallei* (Fig. 2), we suggest that *manC* defines the left side of the capsular gene cluster as depicted in Fig. 1(a).

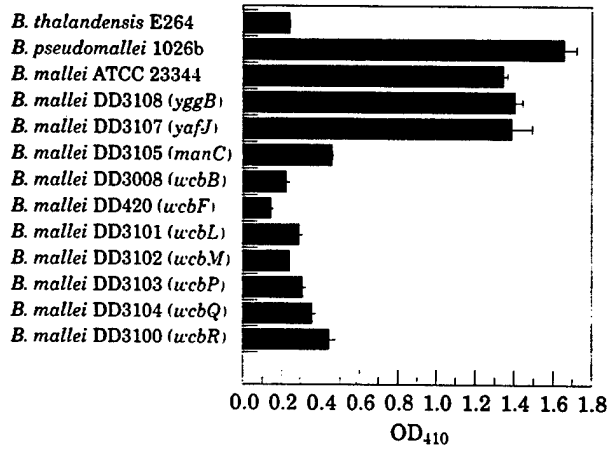


Figure 2. Whole-cell ELISA performed with polyclonal rabbit antiserum directed against the capsular polysaccharide of *B. pseudomallei*. OD_{410}, optical density at 410 nm. Disrupted genes are shown in parentheses following the strain names. The numerical values are the mean of one experiment performed in triplicate ± SD.

IS*407*A is functional in *B. mallei*

In preliminary experiments, we serendipitously isolated a spontaneous capsule mutant after >10 laboratory passages of ATCC 23344. This mutant, termed DD420, did not react with the capsular antibodies in an ELISA (Fig. 2). We cloned and restriction mapped the capsule gene cluster in DD420 and identified a copy of IS*407*A at the 3′ end of *wcbF* at nucleotide position 17 100. There was a 4 bp duplication of the sequence 5′-GCAG-3′ flanking this copy of IS*407*A which is consistent with the generation of 4 bp direct target repeats by the IS3 family of IS elements [23]. These results indicate that IS*407*A is functional in *B. mallei* and that *wcbF* and/or a downstream gene(s) is essential for production of capsular polysaccharide. The relationship, if any, between laboratory passage and IS*407*A insertion in the capsule gene cluster is currently being investigated. However, we did not identify any IS*407*A insertions in the capsule gene cluster when we used animal passaged *B. mallei* for subsequent experiments.

Immunogold electron microscopy of *B. mallei* and *B. thailandensis*

We performed immunogold electron microscopy to see if we could identify a capsular structure on the surface of ATCC 23344, DD3008 and *B. thailandensis* E264. The bacteria were reacted with polyclonal rabbit antiserum directed against the *B. pseudomallei* capsule, washed and reacted with a goat anti-rabbit IgG gold conjugate. ATCC 23344 reacted with the capsular antibodies and formed a thick (approximately 200 nm) and evenly distributed surface layer around the bacteria [Fig. 3(a)]. Similar results were obtained with *B. pseudomallei* 1026b [37]. DD3008, a representative capsule mutant, and *B. thailandensis* E264 did not react with the capsular antibodies [Fig. 3(b) and (c)]. These data directly demonstrate the presence of a capsule on the surface of ATCC 23344 that is immunologically cross-reactive with the *B. pseudomallei* capsular polysaccharide. The isogenic mutant DD3008 and *B. thailandensis* E264 do not produce this capsular polysaccharide.

B. mallei wild type and mutant strains produce LPS O-polysaccharides that are immunologically cross-reactive with those of *B. pseudomallei* and *B. thailandensis*

The LPS O-PS of *B. pseudomallei* and *B. thailandensis* consist of an unbranched heteropolymer with repeating D-glucose and L-talose units with the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- [15, 35–37]. The chemical composition of the *B. mallei* LPS O-PS is currently unknown. We examined the cross-reactivity of whole cell lysates of *B. mallei*, *B. pseudomallei* and *B. thailandensis* with polyclonal rabbit serum raised against a *B. pseudomallei* polysaccharide-flagellin conjugate [38]. As expected, the polyclonal antiserum demonstrated reactivity with the capsule and LPS O-PS of *B. pseudomallei* 1026b in an immunoblot (Fig. 4, lane 3). The LPS O-PS exhibited a typical ladder-like appearance with apparent molecular weights ranging from <30–60 kDa (Fig. 4). The capsular polysaccharide migrated more slowly in the SDS-PAGE gel and was visualized by immunoblot as a smear with apparent molecular weights ranging from 75–>100 kDa. *B. pseudomallei* SRM117, a LPS O-PS mutant [37], reacted with capsular antibodies but not with LPS O-PS antibodies (Fig. 4, lane 4). *B. thailandensis* E264, does not produce a polysaccharide capsule and only demonstrated reactivity with LPS O-PS antibodies (Fig. 4, lane 5). The E264 lysate also contained a band with an apparent molecular weight of 40 kDa that stained with a greater relative intensity. The

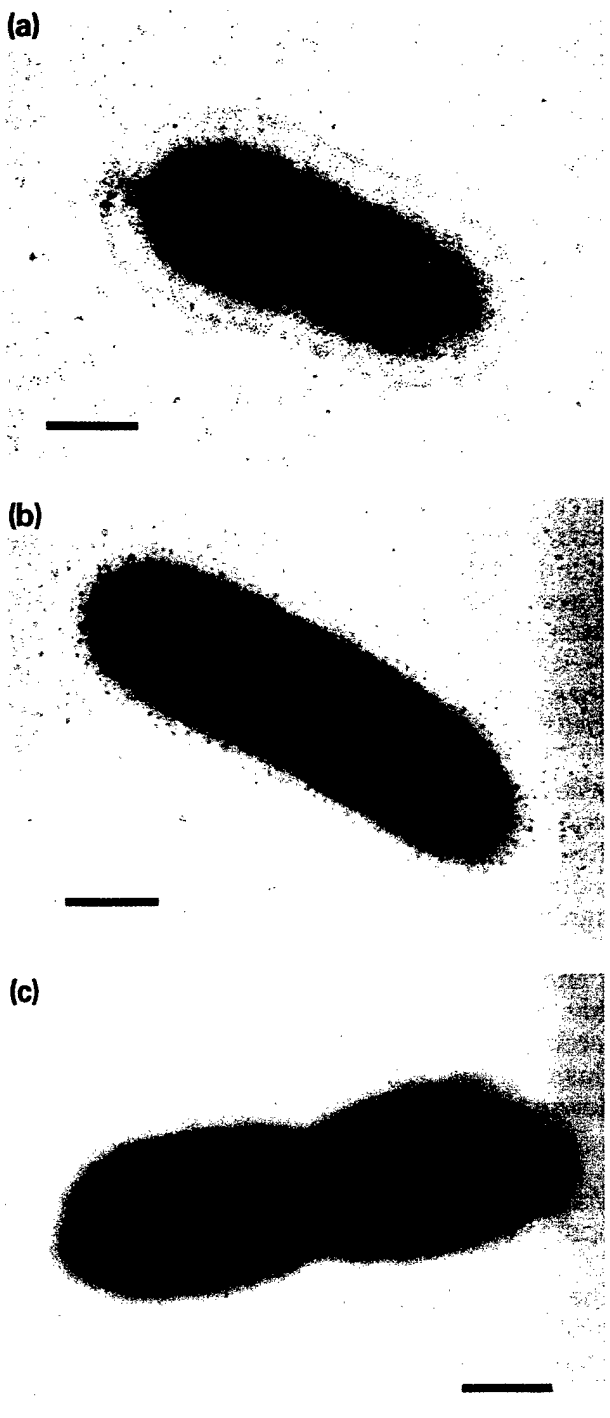


Figure 3. Immunogold electron microscopy of *B. mallei* ATCC 23344 (a), *B. mallei* DD3008 (b) and *B. thailandensis* E264 (c). Bars represent 500 nm.

identity of this product is currently unknown and it is not consistently present in our immunoblots. The capsule and LPS O-PS of ATCC 23344 demonstrated cross-reactivity with the

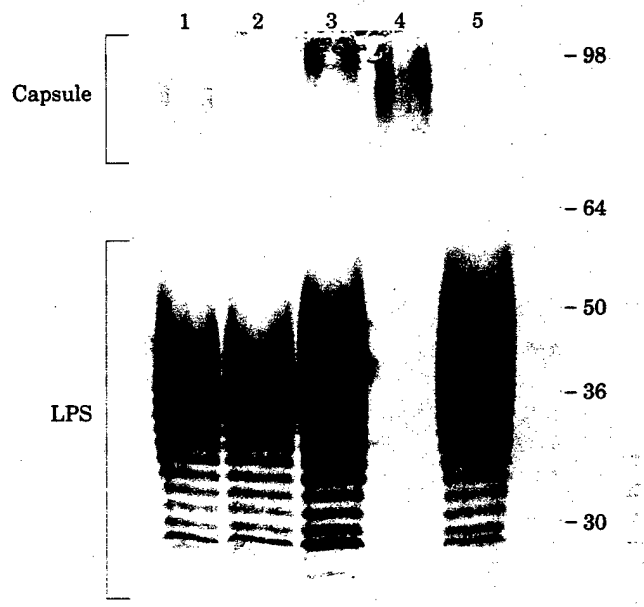


Figure 4. Immunoblot profiles of proteinase K-treated lysates of *B. mallei*, *B. pseudomallei* and *B. thailandensis*. The primary antibody used was polyclonal rabbit serum against a *B. pseudomallei* polysaccharide-flagellin conjugate [38]. The relative positions of capsule and LPS are indicated. Lanes: 1, *B. mallei* ATCC23344; 2, *B. mallei* DD3008; 3, *B. pseudomallei* 1026b; 4, *B. pseudomallei* SRM117; 5, *B. thailandensis*. The apparent molecular weights of the SeeBlue Pre-Stained Protein Standards (Novex) are presented in kDa.

polyclonal antiserum (Fig. 4, lane 1). DD3008 reacted with the LPS O-PS antibodies but not with the capsular antibodies (Fig. 4, lane 2). Similar results were obtained with the other *B. mallei* capsule mutants (data not shown). Taken together, the results demonstrate that *B. mallei*, *B. pseudomallei* and *B. thailandensis* produce LPS O-PS with similar banding patterns and immunological cross-reactivities and that *B. mallei* capsule mutants produce LPS O-PS.

B. mallei DD3008 is avirulent in two animal models of glanders infection

We compared the relative virulence of ATCC 23344 and DD3008 in Syrian hamsters by the intraperitoneal route and in BALB/c mice by the aerosol route. Hamsters are exquisitely sensitive to infection with virulent strains of *B. mallei* via the intraperitoneal route [39, 40]. The 50% lethal doses (LD_{50}s) of ATCC 23344 and DD3008 in the hamster model of infection were <10 and $>10^6$ cfu, respectively. This represents a $>10^5$-fold

difference in virulence between the wild type strain and the capsule mutant. In fact, hamsters infected with DD3008 remained clinically normal throughout the 5 day study. On day 6 of the infection, DD3008-infected animals were killed and their spleens and livers were cultured and tissue samples were fixed and processed for histopathological studies. The spleens and livers were sterile and no characteristic glanders lesions [39] were identified.

Mice were challenged by the aerosol route with *B. mallei* strains using a whole-body aerosol exposure apparatus [41]. The LD_{50}s of ATCC 23344 and DD3008 in the mouse model of infection were 913 and $>10^6$ cfu, respectively. This represents a $>10^3$-fold difference in virulence between the wild type strain and the capsule mutant. All of the DD3008-infected mice remained clinically normal throughout the 21 day study. As DD3008 displayed attenuated virulence in both hamsters and mice, we examined the possibility that it might be useful as an attenuated live vaccine strain. The DD3008-infected mice were rechallenged with approximately 20 LD_{50}s of ATCC 23344 on day 22 and deaths were recorded daily for 21 days. Eighty percent of the mice succumbed to infection after rechallenge with the wild type strain indicating that DD3008 will probably not be useful as an attenuated live vaccine strain. It should be noted that DD3008 grew normally in LB broth containing 4% glycerol.

The capsule gene cluster is also present in *B. thailandensis* but is truncated immediately downstream of *wcbC*$_{Bt}$

We were interested in determining if *B. thailandensis* contained a capsule gene cluster similar to the one present in *B. mallei*. In preliminary experiments, the PCR primers used in the construction of *B. mallei* mutants were used to detect capsule genes in *B. thailandensis*. The genes *dedA*, *yafJ* and *manC* were detected in *B. thailandensis* via the PCR but the genes *wcbM*, *wcbP* and *wcbQ* were not (Table 2). As expected, all of the genes were detected in *B. mallei* (Table 2). In addition, we PCR-amplified a 1.6 kb product from *B. mallei* and *B. thailandensis* with the PCR primers DD14 and DD11, which are specific for *yggB* and *yafJ*, respectively (Table 2). This indicates that the chromosomal arrangement of *yggB* and *yafJ* is conserved between *B. mallei* and *B. thailandensis*. Taken together, these results suggest that the left side of the capsule gene cluster, as depicted in Fig. 1(a), is present in *B. thailandensis*.

We cloned and sequenced 7.9 kb of the *B. thailandensis* capsule gene cluster and identified homologs of *B. mallei manC*, *wcbA*, *wcbB* and *wcbC* [Fig. 5(a)]. The arrangement of the genes were identical in the two species and the percentage nucleotide identities between the *manC*, *wcbA*, *wcbB* and *wcbC* alleles were 80, 55, 72 and 61%, respectively [Fig. 5(a)]. It is interesting to note that *B. thailandensis* contains a 373 bp region that corresponds to the 373 bp subtractive hybridization product from pDD3008 [Fig. 5(a)]. These sequences were only 54% identical and probably contained too many nucleotide differences to allow for efficient "subtraction" to occur using the subtractive hybridization conditions described here. As mentioned above, a gene remnant termed '*manB* was identified immediately downstream of *wcbC*$_{Bm}$ [Fig. 5(a)]. We determined the nucleotide sequence of 1.7 kb downstream of *wcbC*$_{Bt}$ and did not identify a '*manB* gene remnant or a *manB*-like gene. In fact, we did not identify any capsular polysaccharide genes in this region. Fig. 5(b) shows that the *B. mallei* and *B. thailandensis* capsule gene clusters diverge immediately following the *wcbC* stop codons. It should also be noted that there is a significant decrease in the G+C content of the *B. mallei* capsule gene cluster starting near the 3′ end of *wcbC*$_{Bm}$ [Fig. 1(b)]. These results suggest that *B. thailandensis* does not produce a polysaccharide capsule because the capsule gene cluster in this organism is truncated immediately downstream of *wcbC*$_{Bt}$.

Mapping the start site of transcription upstream of *wcbC* using 5′ RACE

The nucleotide sequence of the capsule gene cluster indicates that *wcbB* and *wcbC* are divergently transcribed [Fig. 1(a)]. We performed 5′ RACE (Rapid Amplification of cDNA Ends) in an attempt to identify transcriptional start sites and promoter elements in the 489 bp *wcbB*–*wcbC* intergenic region. The transcriptional start site of *wcbC* was mapped to a G residue 64 bp upstream of the *wbcC* ATG start codon (Fig. 6). We identified sequences that resemble *E. coli* σ^{70} −10 and −35 promoter elements immediately upstream of the *wcbC* start site of transcription (Fig. 6). The spacing between the *wcbC* −10 and −35 promoter elements was 20 bp and there was an "extended −10" motif (TG) [42] 1 bp

Table 2. Detection of capsule genes in *B. thailandensis* via PCR amplification

Gene(s)	PCR primers	Size of PCR product (bp)	PCR amplification results	
			B. mallei	*B. thailandensis*
dedA	DD15/DD16	407	+	+
yafJ	DD11/DD12	571	+	+
manC	DD9/DD10	772	+	+
yggB-yafJ	DD14/DD11	1598	+	+
wcbM	DD1/DD2	396	+	−
wcbP	DD5/DD6	398	+	−
wcbQ	DD7/DD8	650	+	−

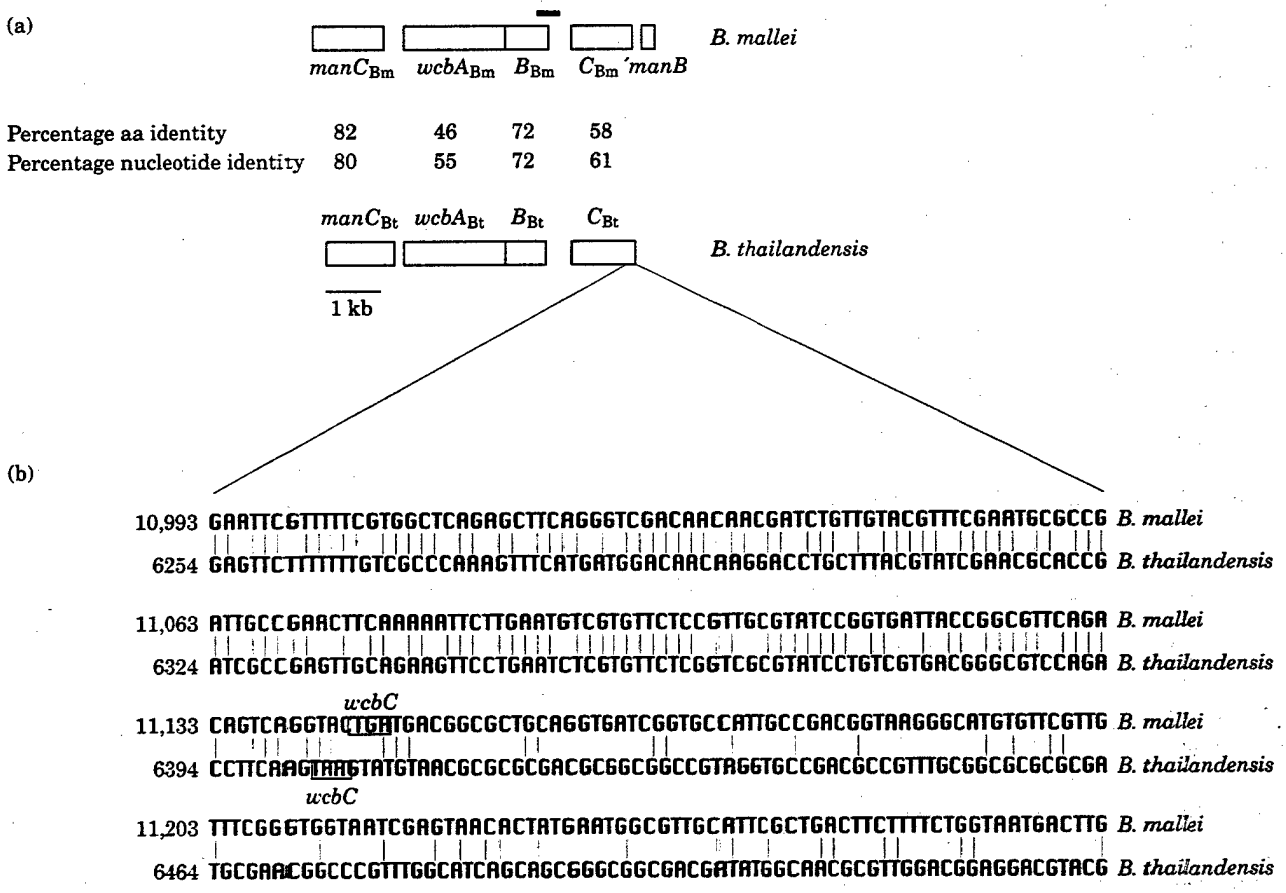


Figure 5. Comparison of the capsule gene clusters in *B. mallei* and *B. thailandensis*. (a) Comparison of *B. mallei* (Bm) and *B. thailandensis* (Bt) *manC*, *wcbA*, *wcbB* and *wcbC*. The boxes represent genes and the gene names are listed below (*B. mallei*) and above (*B. thailandensis*) the corresponding genes. The line above the *B. mallei* gene cluster represents the subtractive hybridization product from pDD3008. The percentage aa identity and percentage nucleotide identity between the *B. mallei* and *B. thailandensis* gene products and genes, respectively, are also shown. (b) Comparison of the nucleotide sequence at the 3′ end and immediately downstream of $wcbC_{Bm}$ and $wcbC_{Bt}$. Nucleotide sequence identities are represented by vertical bars between the corresponding nucleotides and the *wcbC* stop codons are boxed. The numbers to the left of the sequences correspond to the numbering in accession numbers AF285636 (*B. mallei*) and AF285634 (*B. thailandensis*).

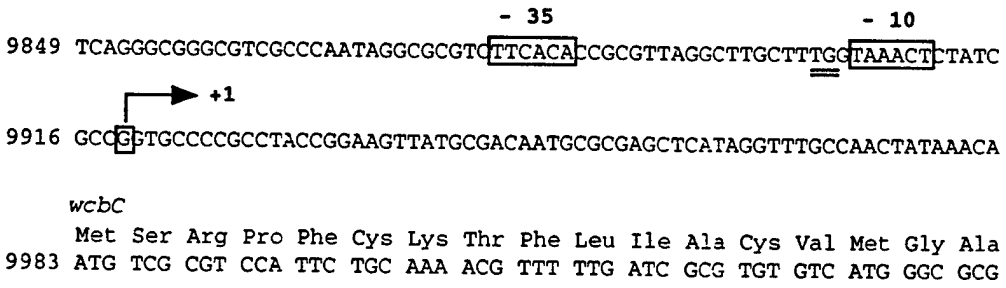


Figure 6. Mapping the transcriptional start site upstream of *wcbC* using 5′ RACE. The transcriptional start site is boxed and indicated by an arrow and a +1. The −10 and −35 sequences of the σ^{70}-like promoter are boxed. The "extended −10" motif, immediately upstream of the −10 region, is double underlined. The first 17 codons of *wcbC* are shown and the corresponding amino acids are presented in three-letter code above the nucleotide sequence. The numbers to the left of the sequences correspond to the numbering in accession number AF285636.

upstream of the −10 promoter element (Fig. 6). Several attempts to identify the transcriptional start site upstream of *wcbB* using 5′ RACE were unsuccessful.

Discussion

In this study, we performed subtractive hybridization between *B. mallei* and *B. thailandensis* and identified a gene cluster encoding a capsular polysaccharide in *B. mallei*. Popov *et al.* detected a capsular structure on the surface of *B. mallei* by ruthenium red staining and electron microscopy, but the chemical structure of the capsule was not determined [43, 44]. The *B. mallei* capsule described here exhibited immunological cross reactivity with antiserum raised against the *B. pseudomallei* capsule [Figs 3(a) and 4]. The *B. pseudomallei* capsule was previously thought to be a glycoprotein, termed surface antigen 8 [34], or a distinct LPS O-PS, termed type I O-PS [35, 36]. Recent work, however, indicates that the homopolymer of 2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose residues is probably a capsular polysaccharide [33]. There is no information on the chemical structure of the *B. mallei* capsule but the results presented here suggest that it is similar, and possibly identical, to the *B. pseudomallei* capsule.

While the pathogens *B. mallei* and *B. pseudomallei* produce a capsule, the closely related nonpathogenic species *B. thailandensis* does not [Figs 3(c) and 4] [14]. Surprisingly, *B. thailandensis* does possess a portion of the capsular gene cluster. A comparison of the *B. mallei* and *B. thailandensis* gene clusters revealed that the *B. thailandensis* gene cluster was truncated immediately following the $wcbC_{Bt}$ stop codon (Fig. 5). There were no polysaccharide genes identified in the 1.7 kb of DNA downstream of $wcbC_{Bt}$ and gapped BLASTX and BLASTP searches [45] with this sequence did not identify any proteins with significant homology in the non-redundant nucleotide and protein databases. The DNA downstream of $wcbC_{Bm}$ in *B. mallei* contains a gene remnant (′*manB*) and a relatively low G+C content as compared to the rest of the *B. mallei* genome (Fig. 1). DNA acquired by lateral transfer often contains a G+C content that is markedly different from the recipient's DNA [27]. It is tempting to speculate that the capsule genes downstream of $wcbC_{Bm}$ were acquired via lateral transfer after the divergence of *B. mallei* and *B. thailandensis* from a common progenitor. Alternatively, the complete capsule gene cluster may have been present in the common progenitor and subsequently lost in *B. thailandensis*. The ′*manB* gene remnant may represent the location where integration or deletion of laterally acquired DNA occurred.

Bacterial group 2 and 3 capsular polysaccharides share similarities in their biosynthesis and in the gene products required for this process [20, 22]. However, group 2 and group 3 gene clusters exhibit differences in gene arrangement and gene content. The *B. mallei* capsule gene cluster most closely resembles a group 3 gene cluster because of its gene arrangement and because it lacks the *kpsF* and *kpsU* homologues that are present in group 2 gene clusters. Group 3 capsule gene clusters are generally organized into three regions [20, 22]. Genes in region 1 and region 3 are involved in capsule export and translocation while genes in

region 2 are involved in biosynthesis of the capsular polysaccharide. The *B. mallei* gene cluster contains region 1 genes (*wbcC*, *wcbD*, *wzm* and *wzt*) but differs from other group 3 capsule gene clusters in that region 3 genes (*wcbA* and *wcbO*) are separated by >17 kb [Fig. 1(a)]. Furthermore, the region 2 genes are not clustered and are not flanked by region 1 and region 3 genes. It is likely that as more group 3 gene clusters are characterized more examples of differences in genetic organization will be revealed. In fact, the region 3 genes in the recently characterized *P. multocida* B:2 group 3 gene cluster are separated by >13 kb [46].

We identified an insertion sequence, IS*407*A, 1.6 kb downstream of *wcbT*. IS*407*A is a member of the IS3 family of insertion sequences [23] and is closely related to *B. cepacia* IS*407* and an IS*407*-like element in *B. pseudomallei* [25, 26]. There are 1–4 copies of this element present in *B. pseudomallei* strains and 8–9 copies in *B. cepacia* strains [25, 26]. We detected >10 copies of IS*407*A in *B. mallei* ATCC 23344 by Southern blotting (data not shown). We also demonstrated that this element is functional in *B. mallei* as it inserted into *wcbF* in the spontaneous capsule mutant DD420. We also identified a *Bordetella parapertussis* IS*1001*-like element [47], termed ISBm*1*, in our *B. mallei-B. thailandensis* subtractive hybridization library (accession number AF285635). To our knowledge, these are the first IS element described in *B. mallei*. Insertion sequences are often present at the boundaries of pathogenicity islands and the *B. mallei* capsule gene cluster possesses most of the criteria of a pathogenicity island as defined by Hacker *et al.* (1997) [48]. IS*407*A may represent the right-hand boundary, as depicted in Fig. 1(a), of the capsule gene cluster as the 1.6 kb of DNA between *wcbT* and IS*407*A does not encode any proteins with significant homology to proteins in the non-redundant sequence databases. Further studies are in progress to determine if the IS*407*A element represents the right-hand boundary of the capsule gene cluster.

Shipovskaya *et al.* described *B. mallei* auxotrophic mutants generated by nitrosoguanidine mutagenesis [49], but there are no published reports describing the construction of genetically-defined mutations in this bacterium. We utilized the mobilizable suicide vector pGSV3 to clone the *B. mallei* capsule gene cluster by plasmid rescue and to construct mutants by plasmid disruption. We constructed nine mutants using the plasmid disruption technique (Fig. 2). These mutants were instrumental in identifying the left-hand boundary of the capsule gene cluster (*manC*) and in identifying seven genes (*manC*, *wcbB*, *wcbL*, *wcbM*, *wcbP*, *wcbQ* and *wcbR*) involved in capsule biosynthesis, export or translocation [Figs 1(a) and 2]. Since it is possible that the mutations in these strains have polar effects on downstream genes, we are currently developing techniques and vectors for the construction of non-polar mutations in *B. mallei* via allelic exchange (gene replacement).

Previous reports have demonstrated that *B. mallei*, *B. pseudomallei* and *B. thailandensis* are closely related serologically [28–32]. The immunoreactive patterns of the LPS molecules from these species were nearly identical (Fig. 4), demonstrating that they produce antigenically related LPS O-PS. Thus, it is likely that the LPS O-PS are responsible, at least in part, for the serological cross-reactivity of these bacteria. *B. pseudomallei* and *B. thailandensis* produce a LPS O-PS that consists of an unbranched heteropolymer with repeating D-glucose and L-talose units [14, 28, 36]. This molecule is required for serum resistance in both *B. pseudomallei* and *B. thailandensis* [36]. Further studies are required to determine the exact chemical structure of the *B. mallei* LPS O-PS and examine its role in serum resistance.

We identified the start site of transcription upstream of *wcbC* using 5′ RACE (Fig. 6). The *wcbC* promoter resembles an *E. coli* σ^{70} promoter in that it contains σ^{70}-like −10 and −35 elements [50, 51]. The optimal spacing between σ^{70} promoter elements in *E. coli* is 17 bp but there are 20 bp separating these elements in the *wcbC* promoter (Fig. 6). The principal sigma factor of *Helicobacter pylori*, σ^{80}, is similar to *E. coli* σ^{70} but exhibits differences in the amino terminus and in region 4.2 [52]. The optimal spacing of the −10 and −35 elements in *H. pylori* σ^{80} promoters is 21 bp [53]. There is currently no information on the principal sigma factor of *B. mallei* and no other promoters have been experimentally identified in this organism. It is possible that the principal sigma factor of *B. mallei* more closely resembles *H. pylori* σ^{80} rather than *E. coli* σ^{70}. It is also interesting to note that the *wcbC* promoter contains an "extended −10" motif (5′-TG-3′) one base upstream of the −10 element (Fig. 6). *E. coli* contains a number of activator-independent σ^{70} promoters that contain the "extended −10" motif [42]. There is no requirement for a −35 element in these promoters as the *E. coli* RNA polymerase σ^{70} subunit makes an alternative

contact with the "extended −10" motif instead [54]. Thus, the spacing between the −10 and −35 elements in the *B. mallei wcbC* promoter may be of no consequence as the *wcbC* promoter may represent an "extended −10" promoter.

Polysaccharide capsules are highly hydrated polymers that mediate the interaction of bacteria with their immediate surroundings [55]. As a result, these surface structures often play integral roles in the interaction of pathogens with their hosts [56]. In this report, we demonstrated that the capsule-negative mutant DD3008 was avirulent in hamsters and mice. There was a >5 log difference in the LD_{50} of ATCC 23344 and DD3008 in hamsters and a >3 log difference in mice. There were no deaths or signs of clinical illness in the animals challenged with DD3008, including those animals that received doses as high as 10^6 cfu. The animal studies demonstrate that the capsular polysaccharide is a major virulence factor of *B. mallei*. The capsule may prevent efficient clearance of *B. mallei* by the host immune system but the specific role(s) of the capsule in the pathogenesis of glanders remains to be elucidated.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 3. *E. coli* was grown at 37°C on LB agar (Lennox L agar) or in LB broth (Lennox L broth). *B. mallei*, *B. pseudomallei* and *B. thailandensis* were grown at 37°C on LB agar or in LB broth containing 4% glycerol. *B. mallei* ATCC 23344 was serially passaged three times in Syrian hamsters and a stock culture was maintained at −70°C by mixing an equal volume of broth culture and 40% glycerol. Unless stated otherwise, all experiments were conducted using animal passaged *B. mallei* ATCC 23344 with limited laboratory subculture. When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin (Ap), 5 μg of gentamicin (Gm), 15 μg of polymyxin B (Pm), 25 μg of streptomycin (Sm), 25 μg of kanamycin (Km) and 15 μg of tetracycline (Tc)/ml for *E. coli* and *B. mallei* and 100 μg of Sm and 50 μg of Tc/ml for *B. pseudomallei* and *B. thailandensis*.

DNA manipulation

Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III Kit (Bio 101, CA, U.S.A.). Chromosomal DNA was isolated from *B. mallei* by using a previously described protocol [57]. Plasmids were purified from overnight cultures by using Wizard *Plus* SV Minipreps (Promega, Madison, WI, U.S.A.).

Subtractive hybridization

Subtractive hybridization was performed using *B. mallei* ATCC 23344 genomic DNA as the "tester" and *B. thailandensis* E264 genomic DNA as the "driver". The protocol described in the CLONTECH PCR-Select Bacterial Genome Subtraction Kit User Manual was followed except that the hybridization temperature was raised from 63 to 73°C. The subtractive hybridization products were cloned into pCR2.1-TOPO and transformed into chemically competent XL10-Gold cells.

Cloning of the *B. mallei* polysaccharide gene cluster

The DNA insert from pDD3008 was released with *Eco*RI and cloned into the corresponding site in the Gm^R suicide vector pGSV3 (Table 3). The resulting plasmid, pGSV3008, was electroporated into *E. coli* S17-1 and the plasmid was conjugated to *B. mallei* ATCC 23344 as described elsewhere [58]. $Gm^R pm^E$ transconjugants were identified after 48–72 h incubation at 37°C. A $Gm^R pm^R$ transconjugant was selected and named DD3008. DNA flanking the site of pGSV3008 recombination in the DD3008 chromosome was isolated by plasmid rescue (self-cloning) [58] with *Bam*HI and *Not*I. The terminal 12 kb on the right side of the polysaccharide gene cluster, as depicted in Fig. 1, was cloned using a similar strategy to that described above but with the plasmid pGSV3104 and the strain DD3104 (Table 3). The *B. thailandensis* gene cluster described in this report was also cloned using a similar strategy with the suicide vector

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
E. coli		
TOP10	General cloning and blue/white screening	Invitrogen
XL10-Gold	General cloning and blue/white screening	Stratagene
S17-1	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Sm^RPm^S	[60]
SM10	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Km^RSm^S	[60]
B. mallei		
ATCC 23344	Type strain; isolated in 1944 from a human case of glanders; Pm^RGm^S	[15]
DD3108	ATCC 23344::pGSV3108; Gm^R	This study
DD3107	ATCC 23344::pGSV3107; Gm^R	This study
DD3105	ATCC 23344::pGSV3105; Gm^R	This study
DD3008	ATCC 23344::pGSV3008; Gm^R	This study
DD420	ATCC 23344::derivative; *wcbF*::IS*407*A	This study
DD3101	ATCC 23344::pDD3101; Gm^R	This study
DD3102	ATCC 23344::pGSV3102; Gm^R	This study
DD3103	ATCC 23344::pGSV3103; Gm^R	This study
DD3104	ATCC 23344::pGSV3104; Gm^R	This study
DD3100	ATCC 23344::pDD3100; Gm^R	This study
B. pseudomallei		
1026b	Clinical isolate; Sm^RTc^S	[58]
SRM117	1026b derivative; *wbil*::Tn5-OT182; does not produce LPS O-PS; Tc^R	[37]
B. thailandensis		
E264	Type strain; soil isolate; Sm^RTc^S	[14]
DD3106	E264::pSKM3106; Tc^R	This study
Plasmids		
pCR2.1-TOPO	3.9 kb TA cloning vector; pMB1 oriR; Km^RAp^R	Invitrogen
pDD3006	pCR2.1-TOPO containing 597 bp subtractive hybridization product corresponding to nucleotide position 13 749–14 341 in *B. mallei* capsule cluster	This study
pDD3008	pCR2.1-TOPO containing 373 bp subtractive hybridization product corresponding to nucleotide position 9341–9713 in *B. mallei* capsule cluster	This study
pDD3023	pCR2.1-TOPO containing 175 bp subtractive hybridization product corresponding to nucleotide position 13 444–13 616 in *B. mallei* capsule cluster	This study
pSKM11	Positive selection cloning vector; IncP oriT; Cole1 ori; Ap^RTc^S	[61]
pSKM3106	pSKM11 containing 772 bp PCR fragment internal to *man*C_{Bt}; Ap^RTc^R	This study
pDD3106H	14.4 kb *Hin*dIII fragment from DD3106 obtained by self-cloning; Ap^RTc^R	This study
pDD3106B	11.1 kb *Bam*HI fragment from DD3106 obtained by self-cloning; Ap^R	This study
pTn*Mod*-OGm′	Minitransposon vector; pMB1 oriR; RP4 oriT; Tn*5* *tnp*; Gm^R	[62]
pGSV3	pTn*Mod*-OGm′ derivative lacking the 1.5 kb *Bgl*II *tnp* fragment; mobilizabile Gm^R suicide vector	This study
pGSV3108	pGSV3 containing 541 bp PCR fragment internal to *yggB*; Gm^R	This study
pGSV3107	pGSV3 containing 571 bp PCR fragment internal to *yafJ*; Gm^R	This study
pGSV3105	pGSV3 containing 619 bp PCR fragment internal to *man*C_{Bm}; Gm^R	This study
pGSV3008	pGSV3 containing 379 bp *Eco*RI fragment from pDD3008; Gm^R	This study
pDD3101	pGSV3 containing 605 bp *Eco*RI fragment internal to *wcbL*; Gm^R	This study
pGSV3102	pGSV3 containing 396 bp PCR fragment internal to *wcbM*; Gm^R	This study
pGSV3103	pGSV3 containing 398 bp PCR fragment internal to *wcbP*; Gm^R	This study
pGSV3104	pGSV3 containing 650 bp PCR fragment internal to *wcbQ*; Gm^R	This study
pGSV3100	pGSV3 containing 506 bp *Eco*RI-*Not*I fragment internal to *wcbR*; Gm^R	This study
pDD3008B	27 bp *Bam*HI fragment from DD3008 obtained by self-cloning; Gm^R	This study
pDD3008N	25.9 bp *Not*I fragment from DD3008 obtained by self-cloning; Gm^R	This study
pDD3104R	15.2 bp *Rca*I fragment from DD3104 obtained by self-cloning; Gm^R	This study

[a] R, resistant; S, susceptible; Sm, streptomycin; Pm, polymyxin B; Km, kanamycin; Gm, gentamicin; Tc, tetracycline; Ap, ampicillin.

Table 4. Oligodeoxyribonucleotides used for PCR amplification of internal gene fragments

Primer	Sequence (5′ to 3′)	Positions	Gene
DD1	ATCCTCTTGACGCGTTTGAG	24 418–24 437	*wcbM*
DD2	AGCAACCTGCGTTGATCAAG	24 814–24 795	*wcbM*
DD5	TTTTGTCGCGAGCATTGACG	27 207–27 226	*wcbP*
DD6	TTGACGAACCCCGGCAAAAC	27 605–27 586	*wcbP*
DD7	ATTCTCGGTCACTTCGATCG	28 535–28 554	*wcbQ*
DD8	CTCGAATACATGCGACAACG	29 185–29 166	*wcbQ*
DD9	TCGATGCATTCGTCGAGAAG	5723–5704	*manC*
DD10	GTGGACTGATTTTCGGTCAG	4952–4971	*manC*
DD11	ATCGATCAGAGCCTGCATTC	4464–4445	*yafJ*
DD12	AACGACGTGCTGTGAAAGAG	3893–3912	*yafJ*
DD13	TGTCGCACTATCTGGAATCG	3426–3407	*yggB*
DD14	CGCAATACACTTGCCAGTAG	2866–2885	*yggB*
DD15	GACGAATGAACGGGATGTTG	2322–2303	*dedA*
DD16	GCGATTCGCTGCTCTTCATC	1915–1934	*dedA*

pSKM11. The plasmid pSKM3106 was recombined into the *B. thailandensis* E264 chromosome and the DNA flanking the recombination site was isolated from DD3106 by self-cloning with *Hin*dIII and *Bam*HI.

Construction of the *B. mallei* mutants

We utilized pGSV3 (Table 3) for constructing *B. mallei* mutants. Briefly, we PCR-amplified internal fragments of the genes to be inactivated and cloned them into pGSV3. The recombinant plasmids were electroporated into *E. coli* S17-1 and conjugated to *B. mallei* ATCC 23344 for 8 h as described elsewhere [58]. Gm^RPm^R transconjugants were identified after a 48–72 h incubation at 37°C. In order to confirm that transconjugants contained the desired mutations, mutated alleles were isolated by self-cloning [58] and sequenced. The genome of each merodiploid strain constructed by this technique contains two copies of the DNA insert separated by pGSV3 DNA (single crossover). The mutants were stable and no revertants were identified in the absence of Gm selection. Chromosomal DNA was PCR-amplified in a 100 µl reaction mix containing 1X *Taq* PCR Master Mix (Qiagen, Valencia, CA, U.S.A.) and 1 µM of each oligonucleotide primer. The thermal cycling parameters used for the PCR were 97°C for 5 min followed by 30 cycles of 97°C for 30 s, 53°C for 30 s and 72°C for 30 s. The nucleotide sequence of the oligodeoxyribonucleotide primers used in the PCR are presented in Table 4.

DNA sequencing and analysis

DNA sequencing was performed by ACGT, Inc. (Northbrook, IL, U.S.A.). DNA and protein sequences were analysed with GenejockeyII software for the Macintosh and the University of Wisconsin Genetics Computer Group Package [59]. The gapped BLASTX and BLASTP programs were used to search the non-redundant sequence database for homologous proteins [45]. The percentage G+C base composition of the 42 146 bp polysaccharide gene cluster was determined using MacVector 6.5 software with a window size of 50.

Enzyme-linked immunosorbent assay (ELISA)

The wells of a round bottom microtitre plate were coated with approximately 5×10^6 bacteria in 100 µl of 0.05 M carbonate buffer pH 9.6, and the plate was incubated for 2 h at 37°C. The wells were washed with PBS plus 0.05% Tween-20 and blocked with a 3% solution of skim milk in PBS-Tween for 1 h at 37°C. The wells were washed and a 1:1000 dilution of a rabbit polyclonal antibody [37] directed against *B. pseudomallei* -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*heptopyranose-(1- was added, and the plate was incubated at 37°C for 1 h. The wells were washed and a 1:1000 dilution of a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO, U.S.A.) was added to each well. The plate was incubated for 1 h at 37°C, washed and developed with the pNPP Liquid Substrate

System (Sigma) for 30 min and the optical density at 410 nm (OD_{410}) was determined.

Immunoblot analysis

Overnight bacterial cultures (50 µl) were centrifuged, washed with PBS and resuspended in 50 µl of sample buffer (4% SDS, 10% glycerol, 5% β-mercaptoethanol and bromophenol blue in Tris buffer, pH 6.8). The samples were boiled for 10 min and treated with proteinase K (25 µg dissolved in 10 µl sample buffer) at 37°C for 1 h. The samples were boiled for 3 min and loaded onto a 12% SDS-polyacrylamide gel. Following electrophoresis, the gel was blotted to an Immuno-Blot PVDF membrane (BIO-RAD, Hercules, CA, U.S.A.) and an immunoassay was performed. The primary antibody, polyclonal rabbit serum raised against a *B. pseudomallei* polysaccharide-flagellin conjugate [38], was used at a 1:250 dilution. The secondary antibody, a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma), was used at a 1:500 dilution. Alkaline phosphatase was detected with SIGMA *FAST* BCIP/NBT substrate solution.

Immunogold electron microscopy

The procedure for immunogold electron microscopy was described previously [37].

Animal studies

Female Syrian hamsters (five per group) were infected by the intraperitoneal route with 10^1, 10^2 and 10^3 *B. mallei* ATCC 23344 and 10^3, 10^4, 10^5 and 10^6 *B. mallei* DD3008. Deaths were recorded daily for 5 days and the LD_{50} values were determined. On day 6 the surviving animals from each group were killed and their spleens and livers were homogenized and cultured. Spleen and liver tissue samples were fixed and processed for histopathological studies as described previously [39].

Female BALB/c mice (10 per group) were challenged by aerosol using a whole-body aerosol exposure apparatus in a Class III safety cabinet in a biological safety level 3 containment facility [41]. While it is not possible to rule out ingestion of bacteria as a result of grooming, histopathological observations suggest that infection initiates in the lung rather than the alimentary canal when using the whole-body aerosol exposure apparatus (D. L. Fritz, unpublished observations). Mice were exposed to aerosols of 92, 1598, 12 425, 124 250 and 461 500 cfu of *B. mallei* ATCC 23344 and 33,285, 9200, 165 600 and 766 667 cfu of *B. mallei* DD3008. Deaths were recorded daily for 21 days and the LD_{50} values were calculated. Mice in the *B. mallei* DD3008 groups were rechallenged with approximately 20 LD_{50}s of *B. mallei* ATCC 23344 on day 22 and deaths were recorded daily for 21 days.

All animals used in this research project were cared for and used humanely according to the following policies: the U.S. Public Health Service Policy on Humane Care and Use of Animals [1996]; the Guide for the Care and Use of Laboratory Animals [1996]; and the U.S. Government Principles for Utilization and Care of Vertebrate Animal Used in Testing, Research, and Training [1985]. All USAMRIID animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal use was approved by the Institutional Animal Care and Use Committee and conducted in accordance with federal Animal Welfare Act regulations.

RNA isolation and 5′ RACE

B. mallei ATCC 23344 was grown to early to middle exponential phase (approximately 5 h) and RNA was isolated using the Promega SV Total RNA Isolation System. 5′ RACE was performed using the GIBCO BRL 5′ RACE System for Rapid Amplification of cDNA Ends. The sequence of the oligodeoxyribonucleotide primer used in first strand cDNA synthesis was 5′-GGAATATTGACATTGC-3′. The sequence of the nested oligodeoxyribonucleotide primer used in PCR amplification of dC-tailed cDNA was 5′-CAGATGGACACCTGAATCG-3′. The 5′ RACE amplification products were cloned into pCR2.1-TOPO and transformed into *E. coli* TOP10 (Table 3).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been deposited in the GenBank database

under the accession numbers AF285636 (*B. mallei*) and AF285634 (*B. thailandensis*).

Acknowledgements

We would like to thank Louise Pitt, Ralph Tammariello, Jim Barth and Marilyn England for assistance in performing the mice aerosol challenges, Kathy Kuehl for electron microscopy assistance and Lynda Miller for necropsy support. We would also like to thank Henry Heine and Joel Bozue for critically reading the manuscript.

References

1 Wilkinson L. Glanders: medicine and veterinary medicine in common pursuit of a contagious disease. *Med Hist* 1981; **25**: 363–84.
2 Alibasoglu M, Yesildere T, Calislar T, Inal T, Calsikan U. Malleus outbreak in lions in the Istanbul Zoo (in German). *Berl Munch Tierarztl Wschr* 1986; **99**: 57–63.
3 McGilvray CD. The transmission of glanders from horse to man. *Can J Public Health* 1944; **35**: 268–75.
4 Neubauer H, Meyer H, Finke EJ. Human glanders. *Revue Internationale Des Services De Sante Des Forces Armees* 1997; **70**: 258–65.
5 Steele JH. Glanders. In: Steele JH, Ed. *CRC Handbook Series in Zoonoses*. Boca Raton, FL: CRC Press, 1979: 339–62.
6 Crohn BB. Notes on blood cultures in human glanders. *proc NY Path Soc* 1908; **8**: 105–10.
7 CDC. Laboratory-acquired human glanders—Maryland, May 2000. *MMWR* 2000; **49**: 532–5.
8 Howe C, Miller WR. Human glanders: report of six cases. *Ann Intern Med* 1947; **26**: 93–115.
9 CDC. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR* 2000; **49 (No. RR-4)**: 1–14.
10 Rosebury T, Kabat EA. Bacterial warfare. A critical analysis of the available agents, their possible military applications, and the means for protection against them. *J Immunol* 1947; **56**: 7–96.
11 Christopher GW, Cieslak TJ, Pavlin JA, Eitzen EM. Biological warfare. A historical perspective. *J Amer Med Assoc* 1997; **278**: 412–7.
12 Wheelis M. First shots fired in biological warfare. *Nature* 1998; **395**: 213.
13 Howe C. Glanders. In: Christian HA, Ed. *The Oxford medicine*. New York: Oxford University Press, 1950: 185–202.
14 Brett PJ, DeShazer D, Woods DE. *Burkholderia thailandensis* sp. nov., description of a *Burkholderia pseudomallei*-like species. *Int J Syst Bacteriol* 1998; **48**: 317–20.
15 Yabuuchi E, Kosako Y, Oyaizu H, *et al.* Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* 1992; **36**: 1251–75.
16 Whitmore A. An account of a glanders-like disease occurring in Rangoon. *J Hyg* 1913; **13**: 1–35.
17 Reeves PR, Hobbs M, Valvano MA, *et al.* Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* 1996; **498**: 495–503.
18 Whitfield C. Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol* 1995; **3**: 178–85.
19 Chung JY, Zhang Y, Adler B. The capsule biosynthetic locus of *Pastuerella multocida* A:1. *FEMS Microbiol Lett* 1998; **166**: 289–96.
20 Clarke BR, Pearce R, Roberts IS. Genetic organization of the *Escherichia coli* K10 capsule gene cluster: identification and characterization of two conserved regions in group III capsule gene clusters encoding polysaccharide transport functions. *J Bacteriol* 1999; **181**: 2279–85.
21 Karlyshev AV, Linton D, Gregson NA, Lastovica AJ, Wren BW. Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. *Mol Microbiol* 2000; **35**: 529–41.
22 Whitfield C, Roberts IS. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol Microbiol* 1999; **31**: 1307–19.
23 Mahillon J, Chandler M. Insertion sequences. *Microbiol Mol Biol Rev* 1998; **62**: 725–74.
24 Wood MS, Byrne A, Lessie TG. IS*406* and IS*407*, two gene-activating insertion sequences from *Pseudomonas cepacia*. *Gene* 1991; **105**: 101–5.
25 Brown NF, Lew AE, Beacham IR. Identification of new transposable genetic elements in *Burkholderia pseudomallei* using subtractive hybridisation. *FEMS Microbiol Lett* 2000; **183**: 73–9.
26 Mack K, Titball RW. The detection of insertion sequences within the human pathogen *Burkholderia pseudomallei* which have been identified previously in *Burkholderia cepacia*. *FEMS Microbiol Lett* 1998; **162**: 69–74.
27 Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. *Nature* 2000; **405**: 299–304.
28 Anuntagool N, Intachote P, Wuthiekanun V, White NJ, Sirisinha S. Lipopolysaccharide from nonvirulent ara$^+$ *Burkholderia pseudomallei* isolates is immunologically indistinguishable from lipopolysaccharide from virulent ara$^-$ clinical isolates. *Clin Diag Lab Immun* 1998; **5**: 225–9.
29 Cravitz L, Miller WR. Immunologic studies with *Malleomyces mallei* and *Malleomyces pseudomallei*. I. Serological relationships between *M. mallei* and *M. pseudomallei*. *J Infect Dis* 1950; **86**: 46–51.
30 Pitt TL, Aucken H, Dance DA. Homogeneity of lipopolysaccharide antigens in *Pseudomonas pseudomallei*. *J Infect* 1992; **25**: 139–46.
31 Stanton AT, Fletcher W. Melioidosis and its relation to glanders. *J Hyg* 1925; **23**: 347–63.
32 Withiekanun V, Smith MD, Dance DAB, *et al.* Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. *J Med Microbiol* 1996; **45**: 408–12.
33 Reckseidler, SL, DeShazer D, Sokol PA, Woods DE. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. *Infect Immun* 2001; **61**: 34–44.

34 Piven NN, Smirnova VI, Viktorov DV, *et al.* Immunogenicity and heterogeneity of *pseudomonas pseudomallei* surface antigen 8 (in Russian). *Zh Mikrobiol (Moscow)* 1996; **4**: 75–8.
35 Knirel YA, Paramonov NA, Shashkov AS, *et al.* Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. *Carbohydrate Res* 1992; **233**: 185–93.
36 Perry MB, MacLean LL, Schollaardt T, Bryan LE, Ho M. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. *Infect Immun* 1995; **63**: 3348–52.
37 DeShazer D, Brett PJ, Woods DE. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Mol Microbiol* 1998; **30**: 1081–100.
38 Brett PJ, Woods DE. Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. *Infect Immun* 1996; **64**: 2824–8.
39 Fritz DL, Vogel P, Brown DR, Waag DM. The hamster model of intraperitoneal *Burkholdera mallei* (glanders). *Vet Pathol* 1999; **36**: 276–91.
40 Miller WR, Pannell L, Cravitz L, Tanner WA, Rosebury T. Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*. II. Virulence and infectivity for animals. *J Bacteriol* 1948; **55**: 127–35.
41 Ivins BE, Fellows PF, Pitt MLM, *et al.* Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. *Vaccine* 1995; **13**: 1779–84.
42 Bown J, Barne K, Michin S, Busby S. Extended −10 promoters. *Nucleic Acids Mol Biol* 1997; **11**: 41–52.
43 Popov SF, Melnikov BI, Lagutin MP, Kurilov VY. Studies of capsule formation of the glanders agent (in Russian). *Mikrobiologicheskii Zhurnal* 1991; **53**: 90–2.
44 Popov SF, Kurilov VY, Yakovlev AT. *Pseudomonas pseudomallei* and *Pseudomonas mallei* are capsule-forming bacteria (in Russian). *J Microbiol, Epidemiol Immunobiol* 1995; **5**: 32–6.
45 Altschul SF, Madden TL, Schaffer AA, *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**: 3389–402.
46 Boyce JD, Chung JY, Adler B. Genetic organization of the capsule biosynthetic locus of *Pastuerella multocida* M1404 (B:2). *Vet Microbiol* 2000; **72**: 121–34.
47 van der Zee A, Agterberg C, van Agterveld M, Peeters M, Mooi FR. Characterization of *IS1001*, an insertion sequence element of *Bordetella parapertussis*. *J Bacteriol* 1993; **175**: 141–7.
48 Hacker J, Blum-Oehler G, Muldorfer I, Tschape H. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* 1997; **23**: 1089–97.
49 Shipovskaya NP, Merinova LK, Ryapis LA. Properties of *Psuedomonas mallei* auxotrohic mutants (in Russian). *J Microbiol, Epidemiol Immunobiol* 1983; **4**: 36–9.
50 Harley CB, Reynolds RP. Analysis of *Escherichia coli* promoter sequences. *Nucleic Acids Res* 1987; **15**: 2343–61.
51 Hawley DK, McClure WR. Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* 1983; **11**: 2237–55.
52 Beier D, Spohn G, Rappuoli R, Scarlato V. Functional analysis of the *Helicobacter pylori* principal sigma subunit of RNA polymerase reveals that the spacer region is important for efficient transcription. *Mol Microbiol* 1998; **30**: 121–34.
53 Vanet A, Marsan L, Labigne A, Sagot M-F. Inferring regulatory elements from a whole genome. An analysis of *Helicobacter pylori* σ^{80} family of promoter signals. *J Mol Biol* 2000; **297**: 335–53.
54 Barne KA, Bown JA, Busby SJW, Minchin SD. Region 2.5 of the *Escherichia coli* RNA polymerase σ^{70} subunit is responsible for the recognition of the "extended −10" motif at promoters. *EMBO J* 1997; **16**: 4034–40.
55 Roberts IS. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu Rev Microbiol* 1996; **50**: 285–315.
56 Moxon ER, Kroll JS. The role of bacterial polysaccharide capsules as virulence factors. *Curr Top Microbiol Immunol* 1990; **150**: 65–85.
57 Wilson K. Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston, RE *et al.*, Eds. *Current Protocols in Molecular Biology*. New York: John Wiley & Sons, 1987: 2.4.1–5.
58 DeShazer D, Brett PJ, Carlyon R, Woods DE. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. *J Bacteriol* 1997; **179**: 2116–25.
59 Devereux J, Haeberli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 1984; **12**: 387–95.
60 Simon R, Priefer U, Puhler A. A broad host range mobilization system for *in vivo* genetic engineering: tranposon mutagenesis in gram negative bacteria. *Bio/Technology* 1983; **1**: 784–91.
61 Mongkolsuk S, Rabibhadana S, Vattanaviboon P, Loprasert S. Generalized and mobilizabile postitive-selection cloning vectors. *Gene* 1994; **143**: 145–6.
62 Dennis JJ, Zylstra GJ. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Appl Environ Microbiol* 1998; **64**: 2710–5.

JOURNAL OF BACTERIOLOGY, Feb. 2002, p. 849–852 Vol. 184, No. 3
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.3.849–852.2002

Molecular and Physical Characterization of *Burkholderia mallei* O Antigens

Mary N. Burtnick, Paul J. Brett, and Donald E. Woods*

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N 4N1

Received 1 August 2001/Accepted 6 November 2001

Burkholderia mallei **lipopolysaccharide (LPS) has been previously shown to cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS; however, we observed that *B. mallei* LPS does not react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O polysaccharide (O-PS). In this study, we identified the O-PS biosynthetic gene cluster from *B. mallei* ATCC 23344 and subsequently characterized the molecular structure of the O-PS produced by this organism.**

Burkholderia mallei is a gram-negative bacterium responsible for a disease known as glanders in solipeds and occasionally in humans (3, 8, 13). The factors involved in the pathogenesis of *B. mallei* infection remain relatively poorly defined at the molecular level. A previous study that identified a polysaccharide gene cluster in *B. mallei* showed that *B. mallei* lipopolysaccharide (LPS) cross-reacts with polyclonal antibodies raised against the LPS of *Burkholderia pseudomallei*, a closely related organism responsible for a disease known as melioidosis (6). In the present study, we investigated the LPS profiles of *B. mallei* strains, identified the gene cluster responsible for O polysaccharide (O-PS) biosynthesis in *B. mallei* ATCC 23344, and determined the physical structure of the *B. mallei* ATCC 23344 O-PS. Additionally, we showed that the O-PS moiety of *B. mallei* LPS is required for resistance to the bactericidal action of serum. Finally, we identified the presence of insertion sequences in two strains of *B. mallei* that disrupt the expression of O-PS.

Analysis of LPS profiles of *B. mallei* strains. The strains and plasmids used in this study are shown in Table 1. The first goal of this study was to assess the LPS profiles of *B. mallei* strains. Initially, we performed Western blot analysis of *B. mallei* ATCC 23344 whole-cell lysates with polyclonal rabbit sera raised against a *B. pseudomallei* bovine serum albumin (BSA)–O-PS conjugate as well as with a *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) according to a previously described protocol (1, 2). As shown in Fig. 1A, *B. mallei* ATCC 23344 reacted with the anti-LPS polyclonal sera, resulting in a typical LPS banding pattern; however, the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) did not react. This indicated that differences exist between *B. mallei* and *B. pseudomallei* O-PS. We further assessed the LPS profiles of 10 different *B. mallei* strains (Fig. 1B). By using Western blot analysis, we showed that 8 of the 10 strains assessed bound the anti-LPS polyclonal sera and displayed typical LPS banding patterns. In contrast, however, two strains, NCTC 120 and ATCC 15310, did not bind the anti-LPS polyclonal sera, as indicated by the absence of bands (Fig. 1B). In order to confirm that the O-PS moiety was absent rather than a different type of O-PS, silver stain analysis was employed. Figure 1C shows the silver stain results confirming that both of these strains lacked O-PS moieties.

Identification and characterization of *B. mallei* ATCC 23344 O-PS biosynthetic gene cluster. In order to investigate the genes responsible for O-PS biosynthesis in *B. mallei*, we constructed a cosmid library by using *B. mallei* ATCC 23344 genomic DNA and the cosmid pScosBC1 by using a previously described protocol (12). Colony hybridizations were then performed with a 1.1-kb DNA fragment containing the recently identified *B. mallei wbiA* gene (P. Brett, M. Burtnick, and D. Woods, unpublished data). Six positive cosmid clones were obtained. Based on the *Bam*HI-*Kpn*I restriction patterns obtained, two cosmid clones, 1C3 and 2B5, were predicted to harbor the entire *B. mallei* O-PS gene cluster. Sequence analysis resulted in 19,918 bp of contiguous sequence containing the entire *B. mallei* O-PS biosynthetic gene cluster with an IS*407*-like insertion sequence element at the 3′ end.

The first 18,738 bp of the *B. mallei* DNA sequence contained 16 predicted ORFs that were identical to those previously defined as the O-PS biosynthetic gene cluster in *B. pseudomallei* (Fig. 2) (5). Sequence alignment of the *B. pseudomallei* and *B. mallei* O-PS biosynthetic regions revealed 99% identity at the nucleotide level. The genes comprising the *B. mallei* O-PS biosynthetic operon were named as per the identical genes found in *B. pseudomallei* (5).

Physical characterization of *B. mallei* O-PS moieties. In order to structurally analyze the *B. mallei* O-PS structure, it was necessary to construct a *B. mallei* strain unable to produce capsular polysaccharide (CPS), because CPS copurifies with LPS. The suicide vector pGSV3008 was employed as previously described to construct *B. mallei* PB100, a derivative of ATCC 23344 that does not produce CPS (6). The O-PS was purified as previously described for *B. pseudomallei*. Figure 3 shows ^{13}C nuclear magnetic resonance (^{13}C-NMR) analysis (Complex Carbohydrate Research Center, University of Georgia, Athens) results demonstrating that the *B. mallei* O-PS

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, 3330 Hospital Dr. NW, Calgary, Alberta, Canada T2N 4N1. Phone: 403-220-2564. Fax: 403-283-5241. E-mail: woods@ucalgary.ca.

TABLE 1. Bacterial strains and cosmids or plasmids used in this study

Strain, cosmid, or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
SM10	Mobilizing strain: transfer genes of RP4 integrated into the chromosome; Km^r Sm^s	11
TOP10	High-efficiency transformation strain with blue/white screening; Ap^s Km^s	Invitrogen
HB101	Serum-sensitive strain	7
B. mallei		
NCTC 120		USAMRIID[a]
NCTC 10248	Human isolate	USAMRIID
NCTC 10229		USAMRIID
NCTC 10260	Human isolate	USAMRIID
NCTC 10247		USAMRIID
ATCC 23344	Human isolate; Pm^r Gm^s	USAMRIID
NCTC 3708	Mule isolate	USAMRIID
NCTC 3709	Horse isolate	USAMRIID
ATCC 10399		USAMRIID
ATCC 15310		USAMRIID
PB100	ATCC 23344::pGSV3008; Pm^r Gm^r	This study
B. pseudomallei 1026b	Clinical isolate; Gm^r Km^r Sm^r Pm^r Tp^s	4
Cosmids		
pScosBC1	Broad-host-range cosmid cloning vector based on pSuperCos1; Ap^r Tp^r	12
p1C3	pScosBC1 from ATCC 23344 library with a 23-kb fragment containing the O-PS biosynthetic gene cluster	This study
p2B5	pScosBC1 from ATCC 23344 library with a 27-kb fragment containing the O-PS biosynthetic gene cluster	This study
Plasmids		
pUC19	Cloning vector with blue/white selection; Ap^r	14
pGSV3008	pGSV containing a 379-bp *Eco*RI fragment from pDD3008, contains internal fragment from the *wcbB* gene; Gm^r	6

[a] U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.

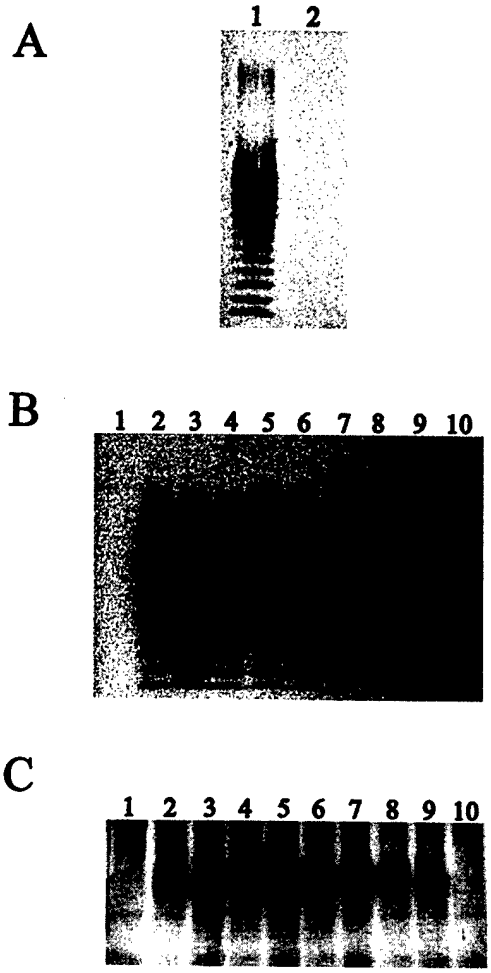


FIG. 1. (A) Western blot analysis of *B. mallei* ATCC 23344. Proteinase K-treated whole-cell lysates were used. In lane 1, the primary antibody used was a 1/2,000 dilution of polyclonal antisera raised against a *B. pseudomallei* BSA–O-PS conjugate, and in lane 2, the primary antibody used was a 1/2,000 dilution of the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W). (B) Western blot profiles of proteinase K-treated whole-cell lysates of *B. mallei* strains. The primary antibody used was polyclonal sera raised against a *B. pseudomallei* BSA–O-PS conjugate. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310. (C) Silver stain analysis of proteinase K-treated whole-cell lysates of *B. mallei* strains. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310.

backbone is similar to that previously described for *B. pseudomallei* O-PS, a heteropolymer of repeating D-glucose and L-talose (9, 10). However, changes are apparent in the O-acetylation pattern of the *B. mallei* L-talose residue in comparison to that of *B. pseudomallei*. Similar to *B. pseudomallei* O-PS, *B. mallei* O-PS demonstrates the presence of *O*-acetyl or *O*-methyl substitutions at the 2′ position of the talose residue. In contrast, *B. mallei* O-PS is devoid of an *O*-acetyl group at the 4′ position of the talose residue. Thus, the structure of *B. mallei* O-PS is best described as 3)-β-D-glucopyranose-(1,3)-6-deoxy-α-L-talopyranose-(1-, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents. Recent studies indicate that the presence of 4-*O*-acetyl groups on the talose residues of *B. pseudomallei* O-PS is due to an O-acetylation locus unlinked to the previously described O-PS biosynthetic operon (Brett et al., unpublished). If this is the case, then the unlinked locus responsible for O-acetylation is either not present or is nonfunctional in *B. mallei* strains. The presence or absence of *O*-acetyl groups on the O-PS moieties may have consequences when O-PS is considered as a component of a vaccine that protects against both *B. mallei* and *B. pseudomallei.*

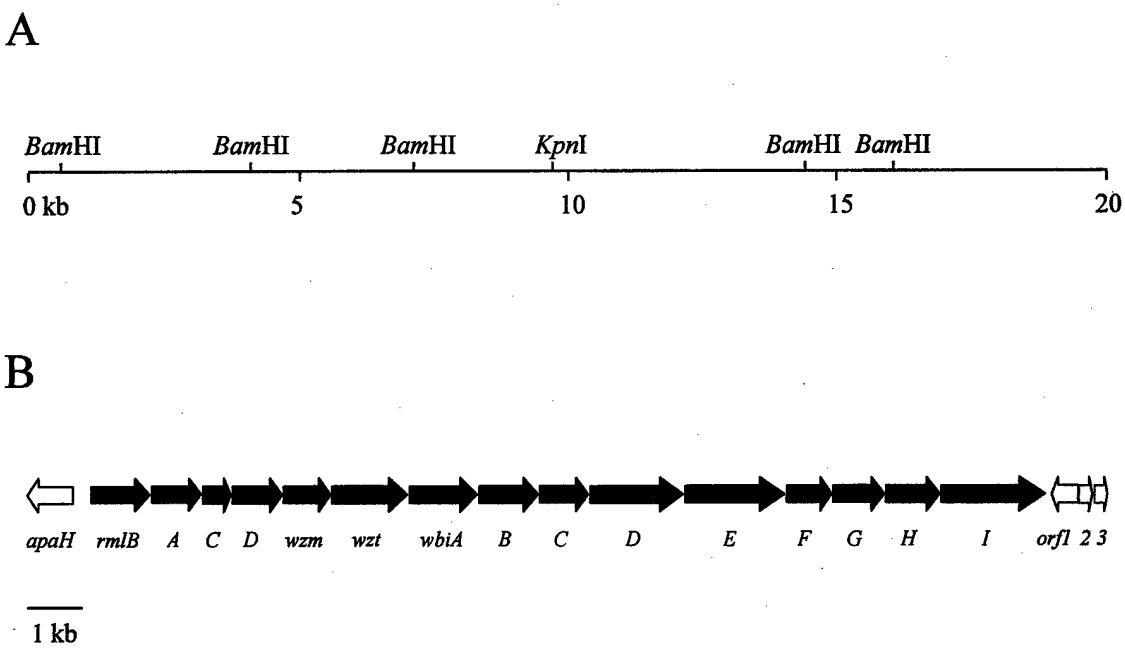


FIG. 2. Restriction and genetic maps of the *B. mallei* O-PS biosynthetic gene cluster. (A) Restriction map. The horizontal line represents the *B. mallei* DNA insert of cosmid 2B5. The locations of *Bam*HI and *Kpn*I cleavage sites used for subcloning are shown. Two additional *Bam*HI sites at the 5′ and 3′ ends of 2B5, which were part of the pScosBC1 vector, are not shown. (B) Genetic map. The location and direction of transcription of the genes are represented by arrows, and the gene names are shown below. The gray arrows indicate the genes involved in O-PS biosynthesis based on homology to the *B. pseudomallei* O-PS biosynthetic operon.

***B. mallei* survives in 30% NHS, and serum-sensitive strains lack the O-PS moiety of LPS.** The ability of *B. mallei* ATCC 23344 to grow in the presence of 30% normal human serum (NHS) was initially assessed with a serum bactericidal assay (5) in which viable counts were determined at 2, 4, 8, and 18 h. *B. mallei* ATCC 23344 was shown to survive in the presence of 30% NHS over the course of 18 h (Fig. 4A). Serum-resistant *B. pseudomallei* 1026b and serum-sensitive *Escherichia coli* HB101 were employed as controls.

In order to assess the role of *B. mallei* O-PS in serum resistance, NHS bactericidal assays (5) were performed with *B. mallei* ATCC 23344 and *B. mallei* NCTC 120 and ATCC 15310, the two strains lacking O-PS. *B. mallei* ATCC 23344 remained resistant to the killing action of 30% NHS, while NCTC 120 and ATCC 15310 were completely killed following a 2-h incubation in 30% NHS (Fig. 4B). The other seven *B. mallei* strains used in this study possessed intact O-PS moieties and were resistant to the bactericidal action of 30% NHS (data not shown). These results suggested that *B. mallei* O-PS moieties play a crucial role in the serum resistance of this organism: this correlates well with previous studies demonstrating that *B. pseudomallei* O-PS is required for serum resistance (5).

Identification of insertion sequence IS*407* in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310. In order to determine if the O-PS biosynthetic gene clusters of NCTC 120 and ATCC 15310 had been disrupted, we chose to individually PCR amplify each gene present in this cluster. Deoxyoligonucleotide primers were designed outside of the 5′ and 3′ ends of each gene. *B. mallei* ATCC 23344 chromosomal DNA was used a control as an indicator of the size of a wild-type copy of each gene. Alterations were observed in the *wbiE* PCR product from NCTC 120 and in the *wbiG* PCR product from ATCC 15310. The PCR products obtained in both cases were approximately 1.5 kb larger than those obtained with ATCC 23344 genomic DNA (data not shown). Cloning and sequence analysis of the NCTC 120 *wbiE* and ATCC 15310 *wbiG* PCR products revealed the presence of insertion sequences within these two genes. In NCTC 120, an IS*407*-like element was located after nucleotide 13615 of the O-PS operon in the *wbiE* gene. In ATCC 15310, an IS*407*-like element was located following nucleotide 15107 of the O-PS operon in the *wbiG* gene. It is likely that the presence of insertion elements in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310 is responsible for the loss of expression of O-PS in these two strains. DeShazer et al. have previously demonstrated the presence of an IS*407*-like element (termed "IS*407A*") at the 3′ end of the CPS gene cluster and have shown that this element is active in *B. mallei* (6). The data presented in this paper certainly support the view that IS*407* is functionally active in *B. mallei*.

This work was funded by the Department of Defense contract no. DAMD 17-98-C-8003 and CIHR MOP 31343. M.N.B. is the recipient of an Alberta Heritage Foundation for Medical Research Studentship Award.

We are grateful to Patricia Baker and Francois Becotte for excellent technical assistance. We thank David DeShazer for providing us with the plasmid pGSV3008.

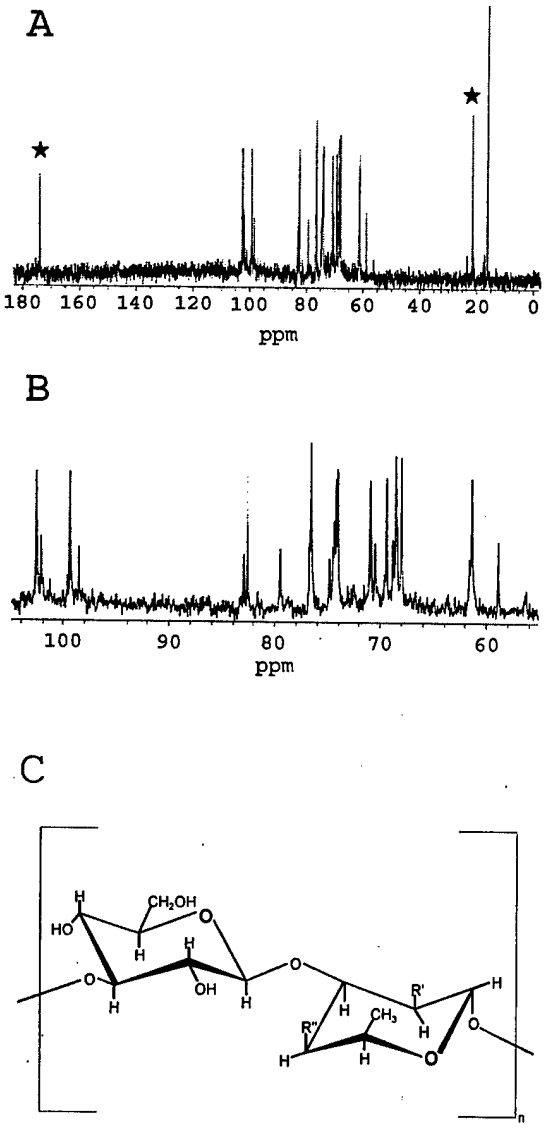


FIG. 3. ^{13}C-NMR analysis of *B. mallei* PB100 O-PS. (A) *O*-Acetyl peaks are indicated by stars. (B) Expanded view of the region running from 100 to 60 ppm. (C) Structure of *B. pseudomallei* and *B. mallei* O-PS. In *B. pseudomallei*, in 33% of the talose residues, R′ = *O*-methyl and R″ = *O*-acetyl, and in 66% of the talose residues, R′ = *O*-acetyl and R″ = OH. In *B. mallei*, R′ = *O*-acetyl or *O*-methyl and R″ = OH.

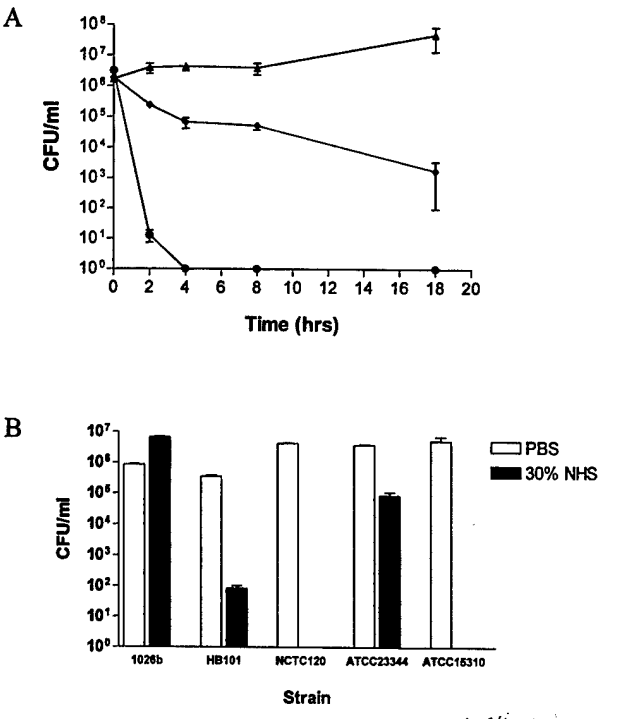


FIG. 4. Serum bactericidal assays with *B. mallei* strains. (A) Thirty percent NHS killing assay in which viable counts were determined at the 2-, 4-, 8-, and 18-h time points. *B. pseudomallei* 1026b (▲), *B. mallei* ATCC 23344 (◆), and *E. coli* HB101 (●). (B) Thirty percent NHS killing assay in which viable counts were determined following a 2-h incubation at 37°C. Control tubes containing phosphate-buffered saline (PBS) are shown as white bars, and experimental tubes containing 30% NHS are shown as gray bars. Error bars indicate standard deviations.

REFERENCES

1. **Brett, P. J., and D. E. Woods.** 1996. Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide–flagellin protein conjugates. Infect. Immun. **64**:2824–2828.
2. **Bryan, L., S. Wong, D. Woods, D. Dance, and W. Chaoagul.** 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of lipopolysaccharide from *Pseudomonas pseudomallei*. Can. J. Infect. Dis. **5**:170–178.
3. **Centers for Disease Control and Prevention.** 2000. Laboratory acquired human glanders—Maryland May 2000. Morb. Mortal. Wkly. Rep. **49**:532–533.
4. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179**:2116–2125.
5. **DeShazer, D., P. J. Brett, and D. E. Woods.** 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol. Microbiol. **30**:1081–1100.
6. **DeShazer, D., D. M. Waag, D. L. Fritz, and D. E. Woods.** 2001. Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microb. Pathog. **30**:253–269.
7. **Fernandez, R. C., and A. A. Weiss.** 1994. Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. Infect. Immun. **62**:4727–4738.
8. **Howe, C., and W. Miller.** 1948. Human glanders: report of six cases. Ann. Intern. Med. **26**:93–115.
9. **Knirel, Y. A., N. A. Paramonov, A. S. Shashkov, N. K. Kochetkov, R. G. Yarullin, S. M. Farber, and V. I. Efremenko.** 1992. Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. Carbohydr. Res. **233**:185–193.
10. **Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho.** 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. Infect. Immun. **63**:3348–3352.
11. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1**:784–791.
12. **Sokol, P. A., P. Darling, D. E. Woods, E. Mahenthiralingam, and C. Kooi.** 1999. Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine N^5- oxygenase. Infect. Immun. **67**:4443–4455.
13. **Wilkinson, L.** 1981. Glanders: medicine and veterinary medicine in common pursuit of a contagious disease. Med. Hist. **25**:363–384.
14. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.

ELSEVIER

FEMS Microbiology Letters 218 (2003) 323–328

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

The *wbiA* locus is required for the 2-*O*-acetylation of lipopolysaccharides expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*

Paul J. Brett, Mary N. Burtnick, Donald E. Woods *

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1

Received 4 September 2002; received in revised form 8 November 2002; accepted 20 November 2002

First published online 7 December 2002

Abstract

Burkholderia pseudomallei and *Burkholderia thailandensis* express similar O-antigens (O-PS II) in which their 6-deoxy-α-L-talopyranosyl (L-6dTal*p*) residues are variably substituted with *O*-acetyl groups at the *O*-2 or *O*-4 positions. In previous studies we demonstrated that the protective monoclonal antibody, Pp-PS-W, reacted with O-PS II expressed by wild-type *B. pseudomallei* strains but not by a *B. pseudomallei wbiA* null mutant. In the present study we demonstrate that WbiA activity is required for the acetylation of the L-6dTal*p* residues at the *O*-2 position and that structural modification of O-PS II molecules at this site is critical for recognition by Pp-PS-W.

Keywords: *Burkholderia* species; O-antigen; Virulence determinant; *trans*-Acylase

1. Introduction

Burkholderia pseudomallei, the etiologic agent of melioidosis, is a Gram-negative bacterial pathogen responsible for disease in both humans and animals [1,2]. Previous studies have demonstrated that the lipopolysaccharide (LPS) expressed by *B. pseudomallei* is both a virulence determinant and a protective antigen [3–6]. Consequently, the O-antigen (O-PS II) has become a significant component of the various sub-unit vaccine candidates that we are currently developing for immunization against melioidosis [7].

The O-PS II moiety produced by *B. pseudomallei* is an unbranched heteropolymer consisting of disaccharide repeats having the structure 3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→ in which ~33% of the 6-deoxy-α-L-talopyranose (L-6dTal*p*) residues possess 2-*O*-methyl and 4-*O*-acetyl substitutions while the remainder of the L-6dTal*p* residues bear only 2-*O*-acetyl modifications [8,9]. Studies have also demonstrated that the non-pathogenic species *Burkholderia thailandensis* synthesizes an O-antigen with the same repeating unit [10]. Recently, we demonstrated that the O-antigen (O-PS) expressed by *Burkholderia mallei*, the causative agent of glanders, is virtually identical to O-PS II except that it lacks acetyl modifications at the *O*-4 position of the L-6dTal*p* residues [11]. Curiously, however, pairwise comparisons between the *B. mallei* and *B. pseudomallei* O-polysaccharide biosynthetic clusters failed to reveal any sequence differences that could account for the structural dissimilarities observed between O-PS and O-PS II [5,11].

In the current study, we used a combination of molecular and physical approaches to further characterize the role of the *wbiA* locus which is thought to be involved in the acetylation of O-PS II antigens [5].

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli*, *B. pseudomallei* and *B. thailandensis* strains were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. *B. mallei* strains were

* Corresponding author. Tel.: +1 (403) 220-2564; Fax: +1 (403) 283-5241.
E-mail address: woods@ucalgary.ca (D.E. Woods).

0378-1097/02/$22.00
PII: S0378-1097(02)01176-X

grown at 37°C in LB broth containing 4% glycerol or on LB agar containing 4% glycerol. For *E. coli*, antibiotics were used at the following concentrations: ampicillin (Ap) 100 μg ml^{-1}, gentamicin (Gm) 15 μg ml^{-1} and kanamycin (Km) 25 μg ml^{-1}. For *B. pseudomallei* and *B. thailandensis*, antibiotics were used at the following concentrations: Gm 25 μg ml^{-1}, streptomycin (Sm) 100 μg ml^{-1} and trimethoprim (Tp) 100 μg ml^{-1}. Bacterial strains were maintained at −70°C in 20% glycerol suspensions.

2.2. DNA manipulations and transformations

Molecular and cloning techniques were performed essentially as described by Sambrook et al. [12]. Plasmids were purified using QIAprep spin plasmid minipreps (Qiagen). Genomic DNA was isolated using the Wizard™ Genomic DNA Isolation kit (Promega). Competent *E. coli* were transformed using standard methods.

2.3. PCR amplification and sequence analysis of wbiA genes

The *wbiA* genes from *B. thailandensis* ATCC 700388 and *B. pseudomallei* 1026b were PCR amplified from purified chromosomal DNA samples using the wbiA-5′ (5′-GCTCTAGACATGAGATCGTGCTTGAGCG-3′) and wbiA-3′ (5′-GGGGTACCGATAAAGCCAGCCCCACCGG-3′) primer pair; the *Xba*I and *Kpn*I sites in the linker regions are underlined. The primers were designed at the 3′-end of *wzt* and the 5′-end of *wbiB* using the previously described *B. pseudomallei* O-PS II biosynthetic gene cluster (GenBank database accession number AF064070). Reactions were performed using *Taq* polymerase (Invitrogen) as per manufacturer's instructions except that the denaturing temperature was increased to 97°C to compensate for the high G/C content of the chromosomal DNAs. The resulting PCR products were then cloned into pCR2.1-TOPO and sequenced on both strands. Sequence analyses were conducted with the aid of DNASIS version 2.5 (Hitachi) as well as the BLASTX and BLASTP programs [13]. The *Shigella flexneri* bacteriophage SF6 *oac* GenBank accession number is X56800. The *B. thailandensis* nucleotide sequence reported in this study was entered into the GenBank database under accession number AY028370.

2.4. Construction and complementation of wbiA mutants

B. pseudomallei PB604, a strain harboring an insertionally inactivated *wbiA* gene, was previously constructed by DeShazer et al. [5]. The *wbiA* gene of *B. thailandensis* was insertionally inactivated using the allelic exchange vector pPB604Tp resulting in strain BT604. Allelic exchange was performed as previously described [5,14]. Mutants were complemented *in trans* using the broad host range vector pUCP31T harboring a wild-type copy of the *B. pseudomallei wbiA* locus. Plasmids were conjugated to *B. pseudomallei* and *B. thailandensis* as previously described [15].

2.5. Western blot and silver stain analysis

Whole cell lysates were prepared as previously described [16] and used in both Western immunoblot and silver stain analyses. Overnight bacterial cultures were pelleted, resuspended in lysis buffer and boiled prior to SDS–PAGE analysis on 12% gels. Immunoblots were performed as previously described [17] using rabbit polyclonal antisera specific for *B. pseudomallei* O-PS II. Silver stain analyses were performed as previously described [18].

2.6. Purification of LPS and O-PS

LPS was purified using a previously described hot aqueous phenol extraction protocol [7,9]. Delipidation of the LPS molecules was achieved via mild acid hydrolysis (2% acetic acid) followed by size exclusion chromatography (Sephadex G-50) as previously described by Perry et al. [9]. Carbohydrate positive fractions were detected using a phenol–sulfuric acid assay [19]. The purity of the carbohydrate preparations was determined to be >90% in all instances. Protein contamination was determined using bicinchoninic acid assays (Pierce) while nucleic acid contamination was estimated from $OD_{260/280}$ measurements.

2.7. Nuclear magnetic resonance (NMR) spectroscopy analysis

^{13}C-NMR spectra were recorded at 100.5 MHz and the chemical shifts were recorded in ppm relative to an internal acetone standard (31.07 ppm [^{13}C]; Spectral Data Services, Champaign, IL, USA).

3. Results and discussion

3.1. Comparison of wbiA alleles from B. thailandensis and B. pseudomallei

The *wbiA* allele from *B. thailandensis* ATCC 700388 was cloned and sequenced as described in Section 2. Analysis of the 1239-bp open reading frame contained within the cloned PCR product demonstrated sequence identities of 93.6% at the nucleotide and 95.0% at the amino acid levels in comparison to the previously characterized *B. pseudomallei* 1026b *wbiA* allele (Fig. 1). Based upon these preliminary results we predicted that the function of WbiA would be similar in both *B. pseudomallei* and *B. thailandensis*.

Further analysis of the *wbiA* gene products expressed by the two *Burkholderia* species demonstrated the presence of conserved amino acid motifs that defines a family of inner membrane *trans*-acylases. The structural and functional significance of these motifs, however, has yet to be determined. The family includes *Salmonella typhimuri-*

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
SM10	Mobilizing strain: expresses RP4 *tra* genes; Km^r Sm^s	[21]
TOP10	High efficiency transformation	Invitrogen
B. pseudomallei		
1026b	Clinical isolate: Gm^r Km^r Sm^r Pm^r Tp^s	[5]
DD503	1026b derivative: Δ(*amrR-oprA*) *rpsL*; Sm^r Pm^r Gm^s Km^s Tp^s	[5]
PB604	DD503 derivative: *wbiA*::*dhfrIIb*-p15A oriV; Tp^r	[5]
PB605	PB604 (pUCP31T); Gm^r Tp^r	This study
PB606	PB604 (p31wbiA); Gm^r Tp^r	This study
B. thailandensis		
ATCC 700338	Type strain (soil isolate): Gm^r Km^r Sm^r Pm^r Tp^s	[10]
DW503	ATCC 700338 derivative: *rpsL*; Sm^r Pm^r Gm^s Km^s Tp^s	[22]
BT604	DW503 derivative: *wbiA*::*dhfrIIb*-p15A oriV; Tp^r	This study
BT605	BT604 (pUCP31T); Gm^r Tp^r	This study
BT606	BT604 (p31wbiA); Gm^r Tp^r	This study
B. mallei		
ATCC 23344	Type strain (human isolate)	USAMRIID[a]
Plasmids		
pCR2.1-TOPO	TA cloning vector: ColE1 ori; Ap^r Km^r	Invitrogen
pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm^r	[23]
p31wbiA	1.37-kb *B. pseudomallei wbiA* PCR product cloned into the *XbaI*/*KpnI* sites of pUCP31T; Gm^r	This study

[a]United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA.

um OafA, *Shigella flexneri* bacteriophage SF6 Oac, *Rhizobium meliloti* ExoZ and *Legionella pneumophila* Lag1 [20]. Interestingly, all are involved in the acetylation of bacterial polysaccharides [20]. A gapped sequence alignment of the WbiA homologues with the Oac *trans*-acylase revealed overall sequence identities of approximately 30% (Fig. 1), a result that is consistent with the family in general.

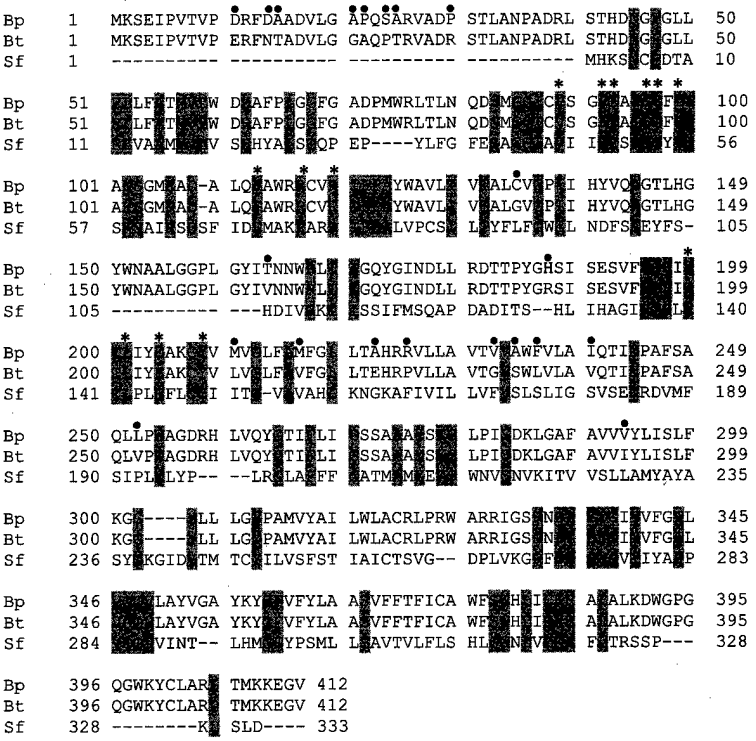


Fig. 1. Amino acid alignment of *B. pseudomallei* 1026b (Bp) *wbiA*, *B. thailandensis* ATCC 700388 (Bt) *wbiA* and *S. flexneri* phage SF6 (Sf) *oac* gene products. Shaded residues represent identity amongst the aligned sequences. Dots indicate dissimilarities between the Bp and Bt proteins. Asterisks indicate residues conserved amongst members of the family of integral membrane proteins involved in the acylation of exported carbohydrates.

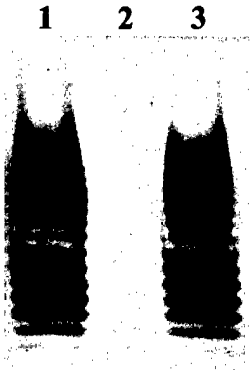


Fig. 2. Western immunoblot analysis of purified *B. thailandensis* LPS antigens. The primary antibody used was the O-PS II specific Pp-PS-W mAb. Lane 1, DW503 LPS; lane 2, BT605 LPS; lane 3, BT606 LPS.

3.2. Phenotypic characterization of *wbiA* null mutants

To determine the effect of the *wbiA* null mutations on the synthesis of O-PS II, *B. pseudomallei* PB604 and *B. thailandensis* BT604 were phenotypically characterized using a variety of genetic and immunological approaches. Silver staining of SDS–PAGE fractionated whole cell lysates demonstrated that BT604 was capable of expressing full-length LPS molecules based upon the presence of a characteristic LPS banding pattern (data not shown). The LPS was also shown to be immunologically similar to that expressed by the type strain and DW503 due to the reactivity of the antigen with the O-PS II polyclonal antiserum (data not shown). Interestingly, however, neither the BT604 whole cell lysates nor the purified LPS molecules reacted with the O-PS II specific monoclonal antibody (mAb) Pp-PS-W suggesting that the *wbiA* locus was required for the expression of a native O-PS II moieties (Fig. 2). By complementing BT604 with the broad host range vector, p31wbiA, we were able to restore the reactivity of the whole cell lysates and purified LPS with the Pp-PS-W mAb (Fig. 2). Similar results were observed for the *B. pseudomallei* strains (data not shown).

3.3. Spectroscopic analysis of the O-polysaccharide antigens

The O-polysaccharides from *B. thailandensis* DW503, BT604 and BT606, *B. pseudomallei* DD503, PB604 and PB606 and *B. mallei* ATCC 23344 were isolated and purified as described in Section 2. The ^{13}C-NMR spectrum of the DW503 antigen demonstrated four anomeric carbon signals between 98.5 and 102.6 ppm, two *O*-acetyl signals at 174.1 and 174.6 ppm (CH_3CO) as well as 21.2 and 21.4 ppm (CH_3CO), two 6-deoxyhexose CH_3 signals at 16.0 and 16.2 ppm and an *O*-methyl signal at 58.8 ppm (Fig. 3A), all of which are consistent with previously published values [9]. Similar spectra were also obtained for the

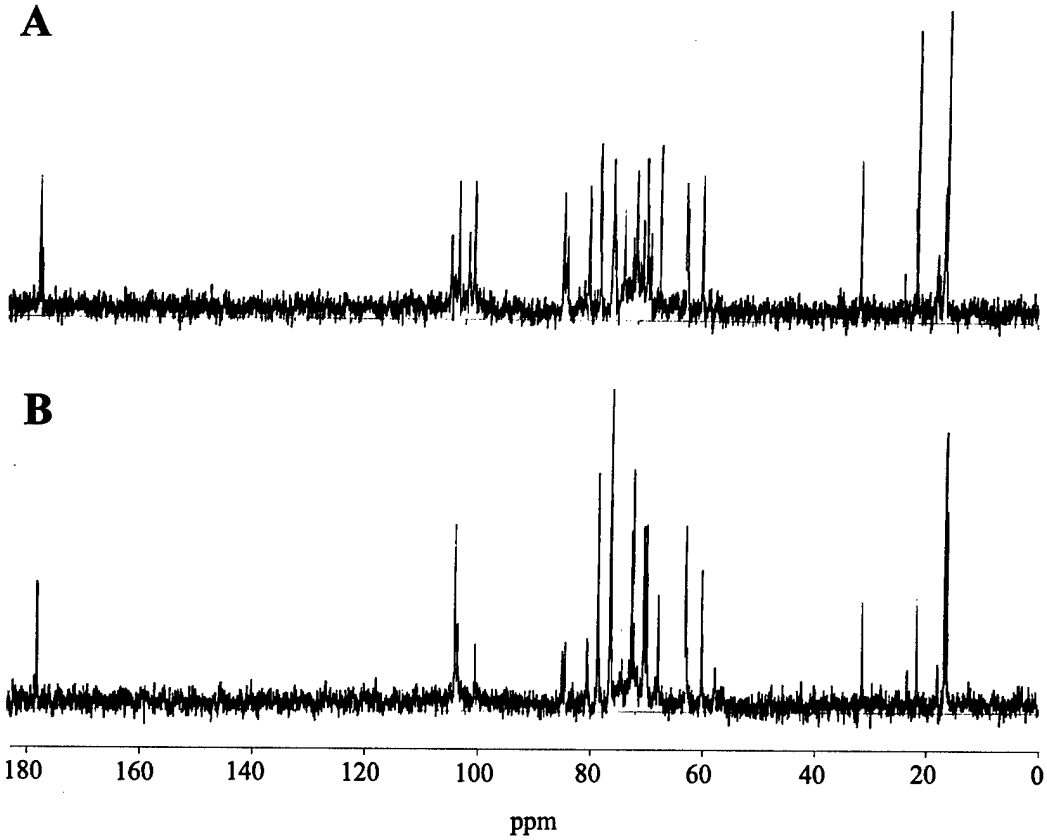


Fig. 3. ^{13}C-NMR spectra of native and mutant O-polysaccharides expressed by *B. thailandensis* strains (A) DW503 and (B) BT604.

BT606, DD503 and PB606 samples (data not shown). In contrast, the ^{13}C-NMR spectrum of the BT604 sample demonstrated four anomeric carbon signals between 98.5 and 102.2 ppm, one *O*-acetyl signal at 174.6 ppm (CH_3CO) and 21.2 (CH_3CO), two 6-deoxyhexose CH_3 signals at 16.0 and 16.3 ppm and an *O*-methyl signal at 58.8 ppm (Fig. 3B). A similar spectrum was recorded for the PB604 sample (data not shown). Based upon these results it was apparent that the O-polysaccharides expressed by BT604 and PB604 were lacking one of the two *O*-acetyl moieties associated with native O-PS II molecules.

To determine which of the *O*-acetyl groups was missing a comparison of the DW503 and BT604 ^{13}C-NMR spectra with the ^{13}C-NMR spectrum obtained for *B. mallei* ATCC 23344 O-PS was conducted. Based upon an analysis of the spectral data we were able to establish that BT604 lacks *O*-acetyl modifications at the *O*-2 position of the L-6dTal*p* residues since O-polysaccharides lacking *O*-acetyl substitutions only at the *O*-4 position would have produced spectra consistent with that obtained for *B. mallei* O-PS (Fig. 4). Similar conclusions can also be drawn for *B. pseudomallei* PB604. Based upon these observations, it is highly probable that a second unlinked locus is responsible for the *O*-acetylation of L-6dTal*p* residues at the *O*-4 position since the *wbiA* locus is the only predicted *trans*-acylase in the O-PS II biosynthetic operon. Studies are currently under way to examine this hypothesis.

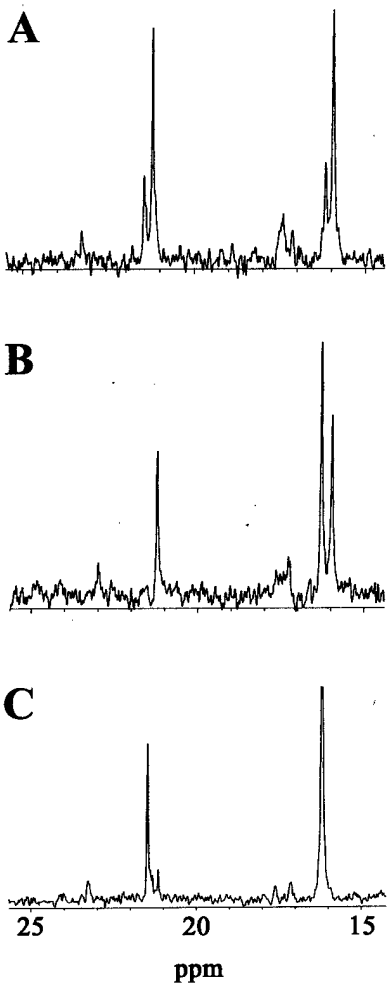


Fig. 4. ^{13}C-NMR spectra of *B. thailandensis* and *B. mallei* O-polysaccharides expanded between the region of 15–25 ppm. (A) DW503, (B) BT604 and (C) ATCC 23344. The peaks around 16 ppm represent 6-deoxyhexose CH_3 signals while those around 21 ppm represent *O*-acetyl (CH_3CO) signals.

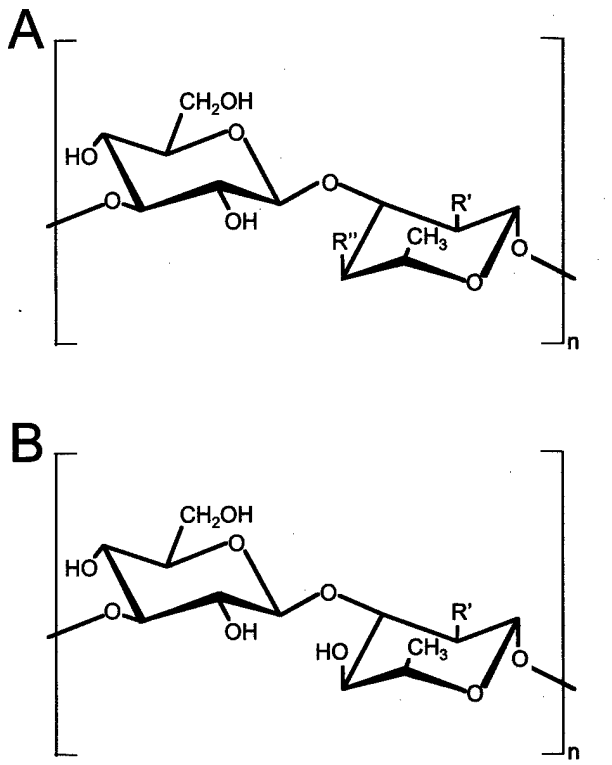


Fig. 5. Structures of (A) *B. pseudomallei* O-PS II and (B) *B. mallei* O-PS. In *B. pseudomallei* R′ = *O*-methyl or *O*-acetyl and R″ = *O*-acetyl or OH. In *B. mallei* R′ = *O*-methyl or *O*-acetyl.

3.4. Characterization of the epitope recognized by the Pp-PS-W mAb

We have recently demonstrated that the O-PS antigen expressed by *B. mallei* does not react with Pp-PS-W [11]. A comparison of the O-antigens expressed by *B. pseudomallei* and *B. thailandensis* with those expressed by *B. mallei* strains suggested that this phenomenon was likely due to differences in the *O*-acetylation patterns exhibited by the O-PS and O-PS II molecules (Fig. 5). Based upon the results of the current study, it is now apparent that the mAb reacts only with 3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→polymers in which the L-6dTal*p* residues are coordinately acetylated at the *O*-2 and *O*-4 positions. Whether or not the 2-*O*-acetyl modification imposes conformational constraints upon the O-polysaccharides or serves more directly as a structural epitope remains yet to be determined. Needless to say, however, these observations have proven to be a valuable re-

minder of the importance of maintaining the structural integrity of O-PS II during the synthesis of the glycoconjugate vaccine candidates.

Acknowledgements

This work was funded by the Department of Defense Contract No. DAMD 17-98-C-8003 and CIHR MOP 31343. M.N.B. was the recipient of an Alberta Heritage Foundation for Medical Research Studentship Award.

References

[1] Dance, D.A.B. (1996) in: Melioidosis (Cook, G.C.E., Ed.), pp. 925–930. W.B. Saunders, London.
[2] Woods, D.E., DeShazer, D., Moore, R.A., Brett, P.J., Burtnick, M.N., Reckseidler, S.L. and Senkiw, M.D. (1999) Current studies on the pathogenesis of melioidosis. Microbes Infect. 1, 157–162.
[3] Charuchaimontri, C., Suputtamongkol, Y., Nilakul, C., Chaowagul, W., Chetchotisakd, P., Lertpatanasuwun, N., Intaranongpai, S., Brett, P.J. and Woods, D.E. (1999) Antilipopolysaccharide II: an antibody protective against fatal melioidosis. Clin. Infect. Dis. 29, 813–818.
[4] Bryan, L., Wong, S., Woods, D., Dance, D. and Chaoagul, W. (1994) Passive protection of diabetic rats with antisera specific for the polysaccharide portion of lipopolysaccharide from *Pseudomonas pseudomallei*. Can. J. Infect. Dis. 5, 170–178.
[5] DeShazer, D., Brett, P.J. and Woods, D.E. (1998) The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol. Microbiol. 30, 1081–1100.
[6] Ho, M., Schollaardt, T., Smith, M.D., Perry, M.B., Brett, P.J., Chaowagul, W. and Bryan, L.E. (1997) Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. Infect. Immun. 65, 3648–3653.
[7] Brett, P.J. and Woods, D.E. (1996) Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. Infect. Immun. 64, 2824–2828.
[8] Knirel, Y.A., Paramonov, N.A., Shashkov, A.S., Kochetkov, N.K., Yarullin, R.G., Farber, S.M. and Efremenko, V.I. (1992) Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. Carbohydr. Res. 233, 185–193.
[9] Perry, M.B., MacLean, L.L., Schollaardt, T., Bryan, L.E. and Ho, M. (1995) Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. Infect. Immun. 63, 3348–3352.
[10] Brett, P.J., DeShazer, D. and Woods, D.E. (1998) *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. 48, 317–320.
[11] Burtnick, M.N., Brett, P.J. and Woods, D.E. (2002) Molecular and physical characterization of *Burkholderia mallei* O-antigens. J. Bacteriol. 184, 849–852.
[12] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[14] Moore, R.A., DeShazer, D., Reckseidler, S., Weissman, A. and Woods, D.E. (1999) Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. 43, 465–470.
[15] DeShazer, D., Brett, P.J., Carlyon, R. and Woods, D.E. (1997) Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. 179, 2116–2125.
[16] Hitchcock, P.J. and Brown, T.M. (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154, 269–277.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Tsai, C.M. and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115–119.
[19] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.
[20] Slauch, J.M., Lee, A.A., Mahan, M.J. and Mekalanos, J.J. (1996) Molecular characterization of the *oafA* locus responsible for acetylation of *Salmonella typhimurium* O-antigen: OafA is a member of a family of integral membrane trans-acylases. J. Bacteriol. 178, 5904–5909.
[21] Simon, R., Priefer, U. and Puhler, A. (1983) A broad host range mobilization system for in vivo genetic eningeering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology 1, 784–791.
[22] Burtnick, M., Bolton, A., Brett, P., Watanabe, D. and Woods, D. (2001) Identification of the acid phosphatase (*acpA*) gene homologues in pathogenic and non-pathogenic *Burkholderia* spp. facilitates TnphoA mutagenesis. Microbiology 147, 111–120.
[23] Schweizer, H., Klassen, T. and Hoang, T. (1996) Improved methods for gene analysis and expression in *Pseudomonas* spp. In: Molecular Biology of Pseudomonads (Nakazawa, T., Furukawa, K., Haas, D. and Silver, S., Eds.). American Society for Microbiology, Washington, DC.